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Assessing the developmental trajectory of mouse models of neurodevelopmental disorders: social and communication deficits in the Neurexin 1α Knockout Mouse.

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Abstract

Neurexin 1α mutations are strongly associated with neurodevelopmental disorders such as autism spectrum disorders and schizophrenia in humans. Studies using the Neurexin 1α knock-out mouse have revealed behavioural abnormalities of relevance to these disorders, and baseline deficits in excitatory synaptic function have been described. However, little is known about the effect of Neurexin 1α deletion on behaviour during development. This study examined the effects of Neurexin 1α deletion on behaviour across a range of developmental time points to determine whether potential abnormalities follow a developmental trajectory. Pups lacking Neurexin 1α emitted a reduced number of ultrasonic vocalisations early in development combined with a restricted repertoire of calls indicative of a loss in complexity in vocal production and showed delays in reaching certain developmental milestones. Behavioural testing revealed that juvenile and adult male Neurexin 1α knock-out mice exhibited social deficits and increased levels of aggression, confirming previous findings. No increases in repetitive behaviours or deficits in motor learning or olfaction were seen. In conclusion this research showed that Neurexin 1α deletion does result in social and communication deficits that follow a developmental trajectory. These are the first experimental data that associate a deletion of Neurexin 1α with alterations in behaviours relevant to autism spectrum disorder across development and highlight the importance of assessing the developmental trajectory in mouse models of neurodevelopmental disorders.

Introduction

Strong evidence exists for a role of synaptic genes in Autism Spectrum Disorders (ASD), including genes encoding the Neurexin family of proteins (Autism Genome Project Consortium in 2007; Kirov et al., 2008). Neurexins are a family of transmembrane proteins located at the pre-synaptic terminal (Ushkaryov et al., 1992). They play an important role in synapse function and development (Missler et al., 2003), which they perform via the formation of cell adhesion complexes with post-synaptic neuroligins and a number of other binding partners (Ichtchenko et al., 1995, Reissner et al., 2013). Neurexin 1, 2 and 3 have been implicated in a wide range of neurodevelopmental disorders including ASD and schizophrenia, for which the strongest evidence of an association appears to be in Neurexin 1α (Gauthier et al., 2011; Grayton et al., 2013). It is estimated that a NRXN1 deletion is present in around 0.32 % of ASD cases, in comparison to only 0.057 % in control cases (Pinto et al., 2014). While this number may seem low, it is important to consider that the most highly associated genes are only present in around 1-2 % of individuals with ASD (Betancur, 2011; Huguet et al., 2013) due to the high levels of genetic heterogeneity, meaning that NRXN1 deletions are in fact among the most common mutations in ASD.

Previous studies using a mouse model for the NRXN1 copy number variation (CNV) mutation, the Neurexin 1α knockout (*Nrxn1α* KO) mouse maintained on a mixed genetic background, found significant deficits in prepulse inhibition, a measure of sensory motor gating, increased repetitive behaviours (self-grooming) and impaired nest building behaviours, that have been related to social behaviour (Etherton et al., 2009). In addition, increased responsiveness to novelty and accelerated habituation to novel environments were observed in male but not female *Nrxn1α* HET mice (Laarakker et al., 2012) and deficits in social memory have been seen in both male and female *Nrxn1α* HET mice (Dachtler et al., 2015). Given the confounding effect of genetic background on observed phenotypes (Wolfer et al., 2002, Schalkwyk et al., 2007), *Nrxn1α* KO mice were generated on a pure genetic background (C57BL/6J). In *Nrxn1α* KO male mice bred on a pure genetic background, altered social approach, reduced social investigation, increased aggression and increased anxiety was observed (Grayton et al., 2013). Together, all these studies suggest that deletions in Neurexin 1α could be responsible for the behavioural differences seen in individuals with ASD. However, none of these studies have tested whether there is a developmental trajectory for any of these behaviours or established a neurodevelopmental basis for the effect of the Neurexin 1α mutation.

A range of social and communication behaviours emerge early in mice and display a clear trajectory which can be measured throughout their development. Ultrasonic vocalisations (USVs), developmental milestones and spontaneous motor behaviours can be investigated from birth up to around two weeks (Fox, 1965; Scattoni et al., 2008; Romano et al., 2013) and juvenile social behaviours

can be tested from around three weeks and through into adulthood (McFarlane et al., 2008). Studying behaviour over a developmental time course could provide evidence of a developmental trajectory and is particularly relevant to the study of neurodevelopmental disorders such as ASD. In addition, studying behaviour through development may allow the actual processes underlying these disorders to be uncovered rather than trying to find them once they have become hidden behind the biological or behavioural processes that may have been triggered in response to the primary processes. However, many published studies on these disorders have focused only on adult mouse behaviour.

In the current study, male and female *Nrxn1α* WT, HET and KO mice were tested across development using a battery of tasks in order to perform a detailed characterisation of mouse behaviour, building on the findings of previous research. Firstly, ultrasonic vocalisations, developmental milestones and motor profile were tested. Subsequently, social, olfactory, motor and repetitive behaviours were assessed in juvenile and adult mice. We hypothesised that social and behavioural deficits may be present in *Nrxn1α* KO mice and may appear from an earlier age than previously reported, possibly revealing a developmental trajectory for ASD-like behaviours in mice.

Materials and Methods

Mouse Generation, Breeding and Husbandry

Male and female *Nrxn1α* KO mice were generated as described (Missler et al., 2003) and were genotyped by PCR using genomic mouse DNA from ear punches (WT primer: CGA GCC TCC CAA CAG CGG TGG CGG GA, KO primer: GAG CGC GCG CGG AGT TGT TGA C, common primer: CTG ATG GTA CAG GGC AGT AGA GGA CCA). These mice had previously been maintained on a C57BL6/SV129 mixed genetic background (Etherton et al., 2009). In order to transfer the KO allele onto a pure C57BL/6J genetic background, mice were subjected to 8 generations of backcrossing to C57BL/6J mice. From the offspring of the F8 pairing, *Nrxn1α* HET mice were crossed together to generate the test mice (*Nrxn1α* WT (+/+), *Nrxn1α* HET (+/-) and *Nrxn1α* KO (-/-) mice).

All mice were housed in Techniplast cages (32cm x 16cm x 14cm) with sawdust (Litaspen premium, Datesand Ltd, Manchester, UK) and basic cage enrichment, consisting of sizzlenest (Datesand Ltd, Manchester, UK) and a cardboard shelter (LBS Biotech, Horley, UK). Cages were never cleaned the day before, or on the day of testing in order to minimise the potential effects of cage disturbance on the behaviour of the mice. All mice had *ad libitum* access to water and food (Rat and Mouse No. 3 Diet (RM3) for breeders and No. 1 (RM1) for test mice; Special Diet Services, Essex, UK). The housing room was maintained at constant room temperature (~21 °C) and humidity (~45%) and kept under a regular light/dark schedule with lights on from 08:00 to 20:00 hours (light = 270 lux). Test

mice were singly housed when weaned. This has been shown previously to have minimal effects on C57BL/6J mice and also eliminates the potential confounds of group housing, such as the establishment of social hierarchies (Brown, 1953; Lad et al., 2010). The oestrous phase of the female mice was not checked in this study, but it is unlikely that this affected the results as there were no major differences in the variance observed in the behavioural measures between males and females. C57BL/6J male and female conspecifics for social tests (juvenile play, social investigation, 3-chamber social approach) were purchased from Charles River (Margate, UK) one week before testing to allow for a habituation period. These conspecific mice were pair-housed by sex and kept in different holding rooms at all times to prevent any exposure to the test animals before social testing.

All housing and experimental procedures were performed in accordance with the U.K. Home Office Animals Scientific Procedures Act 1986, and the work was carried out under licence. All efforts were made to keep animal suffering to a minimum and to reduce the number of animals used.

Ultrasonic vocalisations (USVs) and spontaneous motor behaviours

Ultrasonic vocalisations (USVs) and spontaneous movements were recorded in pups across 3-minute sessions in response to social separation from the mother and siblings at postnatal day (PND) 2, 4, 6, 8 and 12, in a dimly lit (< 10 lux) soundproof chamber. The tattooing for early identification of the mice was carried out on PND 2. USV testing was performed on a batch of 112 mice (Males; 15 WT, 26 HET, 11 KO and Females; 18 WT, 32 HET, 10 KO). An Ultrasound Microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany), sensitive to frequencies of 10–180 kHz, was placed through a hole in the middle of the cover of the sound-attenuating box, about 20 cm above the pup in its glass's container. Vocalisations were recorded using Avisoft Recorder software (Version 3.2). For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.40) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated at a frequency resolution of 488 Hz and a time resolution of 1 ms. Acoustical parameters that were analysed for each test day included: number of calls, duration of calls, frequency and amplitude of the sound at the maximum of the spectrum, as described previously (Scattoni et al., 2008). Moreover, every USV emitted was classified in distinct categories based on internal pitch changes, lengths and shapes, using our previously published categorization (Romano et al., 2013).

For analysis of spontaneous movements, frequency and duration of behavioural items were analyzed by an observer blind to mouse genotype using the NOLDUS OBSERVER software V 10XT (Noldus Information Technology, Wageningen, NL, USA). Consistent with previous studies focused on neonatal rodent behaviour (Romano et al., 2013) the following behavioural patterns were scored: locomotion (general translocation of the body of at least 1 cm in the glass container), immobility (no visible

movement of the animal when placed with all the four paws on the floor), side (no visible movement of the animal when laying on the side), head rising (a single rising of the head up and forward), head shaking (a single lateral displacement of the head), face washing (forepaws moving back and forth from the ears to the snout and mouth), wall climbing (alternating forelimb placing movements on the wall of the container), pivoting (locomotor activity involving the front legs alone and resulting in laterally directed movements), circling (circular locomotor activity involving the all legs and resulting in laterally directed movements), and curling (roll, vigorous side-to-side rolling movements while on the back; curl, a convex arching of back while on side or back, bringing head in a closer opposition to hump/hindlimb region). Body temperature, body weight and righting reflex of pups were measured after vocal and motor recording on PND 2, 4, 6, 8 and 12. Influence of body weight and body temperature on neonatal vocal emission was investigated using a linear mixed-effect model.

Developmental Milestones

Before weaning, a number of developmental milestones can be observed in order to assess physical development along with the development of motor and sensory abilities (Fox, 1965). These were performed on a separate cohort of mice (Males; 8 WT, 24 HET, 7 KO and females; 16 WT, 21 HET, 6 KO). Separate batches of mice were used as it has been shown that excessive handling and maternal separation at an early stage can affect subsequent behavioural development in mice (Flanigan and Cook, 2011; Luchetti et al., 2015). Developmental milestones were assessed at PND 5, 7, 9, 11, 13 and 15, see Table 1 (Heyser et al., 1995; Picker et al., 2006; Ricceri et al., 2007; García-Palomares et al., 2009 and Silverman et al., 2010). Beyond the age of 15 days mice become very jumpy and certain tests become too difficult to accurately assess. Therefore, this was chosen as the final point. To avoid inter-observer variability, the same experimenter recorded all developmental milestone behaviours. Tattooing (coloured tattoo ink, Vet Tech Solutions Ltd, Congleton, Cheshire, UK) for identification was carried out at PND 3 by inserting the ink subcutaneously through a 0.3 mm hypodermic needle into the centre of the paw. Earmarks were collected at PND 10 for later identification since as animals grow older, the pigment of their skin darkens, and the tattoo is harder to see. The experimenter was blind to the genotype throughout testing. Before handling each litter, a clean pair of gloves were put on and rubbed with sawdust from the home cage. This was done to minimise unfamiliar smells of the pup and avoid the mother rejecting the offspring.

Juvenile and Adult Behavioural Testing in *Nrxn1α* mice

All behavioural tests were performed in the light phase between 09:00 and 18:00 hours. For all experiments, the experimenter was blind to the genotype. Behavioural testing was performed using

two batches of mice only (separate to those used in the early USV testing and developmental milestones). This was to ensure a sufficient number of mice in each genotype group per sex were tested while also keeping the possible confounding batch effects to a minimum. Combining the numbers from both batches, 100 animals were tested (Males; 13 WT, 20 HET, 18 KO and Females; 16 WT, 19 HET, 14 KO). Behavioural testing began at PND 30, when mice are still considered to be juvenile. This is also consistent with the age of testing in previous studies carried out at the Institute of Psychiatry, Psychology and Neuroscience (King's College London) on *Nrxn1α* mice by Grayton et al. (2013). The first three tests were juvenile play, juvenile 3-chamber social approach and rotarod. These tests were carried out before 8 weeks of age. From 8 weeks of age, mice are considered to be adult. At this point the experiments were run in the following order up to the age of 12 weeks: adult social investigation, modified adult 3-chamber social approach, marble burying and olfactory habituation. Olfactory habituation/dishabituation test was conducted on one batch of adult mice only. At least one day intertrial interval was included between different tests. All tests were recorded using a camera positioned above the test arenas and movement of each mouse tracked using EthoVision software (Noldus Information Technologies, Wageningen, The Netherlands; http://www.noldus.com/site/doc200403002). After each trial, boli and urine were removed from the test arena which was then cleaned with 1 % Anistel® solution. At the end of testing, mice were returned to their home cage which was returned to the housing room. Light levels for each task varied according to the specific task.

Juvenile Play

Mice were weaned and singly housed at PND 29, 24 hours prior to testing. Each mouse was tested at PND 30 as described previously (McFarlane et al., 2008). Testing was conducted in a dimly lit room, illuminated with red light from 4 cluster lights only (LED cluster red light No. 310-6757; RS Components Northants, UK) with a wavelength of approximately 705 nm. Each test mouse was placed in a clean test cage (Techniplast cage, 32cm x 16cm x 14cm), with a 2 cm thick layer of clean sawdust and habituated to the environment for 5 minutes before the addition of a novel, age- and sex- matched conspecific mouse. A Perspex lid was placed on top of the cage, to contain the mice and to allow the interaction to be recorded. The tail of the conspecific mouse was marked using a permanent marker pen (Pentel, UK) before each test to aid identification. Interactions initiated by the test mouse were recorded for 10 minutes and social play behaviour was later scored from the recordings by researchers who were blind to the mouse genotypes. Details of the behavioural measurers scored in this task are listed in Table 2 (Terranova & Laviola, 2005). Following testing, the test and conspecific mice were returned to their respective home cages and housing rooms.

Table 1. Items assessed in the developmental milestone screen battery.

DEVELOPMENTAL MILESTONE SCREEN		
Test/Observation	**Typical emergence (PND range)**	**PNDs Scored**
Body Weight (g)	**-**	**3,5,7,9,11,13,15**
Body Length (cm)	**-**	**3,7,11**
Tail Length (cm)	**-**	**3,7,11**
Fur Appearance: (0 – absent, 1 – present)	**9 (3-15)**	**3,5,7,9,11**
Eye Opening: complete opening of both eyelids (0 – absent, 1 – present)	**12 (7-17)**	**7,11,13,15**
Ear Canal Opening: complete permeation of the auditory conduct (0 – absent, 1 – present)	**15 (10-20)**	**7,11,13,15**
Incisor eruption: scruff the mouse looking for the teeth appearance (0 – absent, 1 – present)	**7 (5-10)**	**7,9,11,13,15**
Head elevation: (0 – absent, 1 – present)	**12 (9-21)**	**7,9,11,13,15**
Forelimbs and shoulder elevation: (0 – absent, 1 – present)	**7 (5-15)**	**5,7,9,11,13,15**
Surface righting reflex: The pup is placed on its back and its ability to right itself is scored, with a 60-second cut-off (0 – absence of response; 1 – vigorous, but unsuccessful attempts to right; 2 – almost complete response, with one paw still underneath the body; 3 – full response)	**5 (1-10)**	**3,5,7,9,11**
Tactile startle reflex: An air puff is directed towards the pup, whose startle response is recorded (0 – absent, 1 – present)	**11 (3-20)**	**9,11,13,15**
Auditory startle reflex: A tone is presented directly at the pup, and its startle response is recorded (0 – absent, 1 – present)	**15 (11-21)**	**9,11,13,15**
Grasp reflex: The pup's ability to grasp a blunt metal dissecting rod that is stroked against its forepaw (0 – absent, 1 – present)	**7 (3-15)**	**3,5,7,9**
Horizontal screen test: The pup is pulled along a wire mesh that is held horizontally; its ability to grasp it is recorded (0 – no response; 1 – animal grasps the mesh)	**10 (3-18)**	**5,7,9,11,13,15**
Vertical screen test: The pup is pulled along a wire mesh that is held at 45°; its ability to grasp it and begin to climb is recorded (0 – no response; 1 – animal grasps the mesh)	**10 (3-18)**	**5,7,9,11,13,15**
Negative geotaxis: The pup is placed on an inclined wire mesh (45°) with its head facing down; ability to change its orientation and start walking upwards is recorded with a 60-second cut-off (0 – no response; 1 – animal almost succeeds in changing orientation; 2 – animal changes orientation and starts climbing upwards)	**7 (3-15)**	**5,7,9,11,13,15**
Cliff avoidance: The pup is placed on the edge of a cliff, with its forepaws and the head over the edge; the response is positive if the pup turns and crawls away from the cliff (0 – absent; 1 – present)	**8 (2-12)**	**13,15**
Quadrupled walking: The pup is able to walk over a distance exceeding its body length (0 – absent; 1 – present)	**13 (8-18)**	**9,11,13,15**

3-chamber Social Approach (Juvenile Mice)

The 3-chamber social approach is an assay of mouse sociability and preference for social novelty (Yang, Silverman, & Crawley, 2011). Sociability is defined as the subject mouse spending more time in the chamber containing the mouse than in the chamber containing the object (Yang, Silverman & Crawley,

2011). This task was carried out as in Grayton et al. (2013). During testing, the subject mouse is presented with the choice of spending time with either a novel mouse or a novel object. This test can be performed as either two or three trials. Due to time constraints, the batches of mice used were subjected to two trials only.

Table 2. Juvenile Play Behaviours. The number, latency and duration of each behaviour were recorded.

JUVENILE PLAY BEHAVIOURS		
Investigative behaviours	Social sniffing	Sniffing of the body above the shoulders
	Following	Behaviour where the mouse moves in close proximity to the other mouse without making direct contact with the mouse
	Mutual circle	Partners are mutually sniffing each other's anogenital region, while moving in tight circles together with their reciprocal following movements
Affiliative behaviours	Social grooming	Mice groom each other
	Social rest	Test animal is being groomed by the conspecific
	Push under	Test animal pushes its own snout or the whole anterior part of its body under the conspecific's body, and rests for at least 3 s
	Social inactive	Test animal is lying flat or standing still (eyes closed or open) while maintaining close physical contact with the conspecific
Play-soliciting behaviours	Push past	Test animal passes between the wall of the cage and the body of the conspecific by pushing its own body through the narrow space available
	Crawl under	Test animal crawls underneath the conspecific's body, crossing it transversely from one side to the other
	Crawl over	Test animal crawls over the conspecific's body, crossing it transversely from one side to the other

Mice were tested at approximately PND 37. This test was performed using a three chambered apparatus (rectangular clear perplex 3-chambered box, with each chamber 20 cm length x 40.5 cm width x 22 cm height). The box was dimly lit from below (10 lux), and small openings in the dividing walls allowed for easy movement between the chambers. Each chamber was filled with sawdust up to approximately 1 cm. The first trial is a 10 min habituation phase during which the mouse is able to freely explore the 3-chamber apparatus. At the same time, an age and sex-matched novel conspecific

is habituated to sitting under a wire cup in a separate room. After this habituation, the test mouse was briefly confined to the centre chamber while a novel object (a black tally counter resembling a mouse in size and shape) and the conspecific mouse were added to the outer chambers. The chamber location of the novel mouse was counterbalanced across all trials to minimize any potential confound due to a preference for chamber location. During the second 10-minute trial, when presented with the choice of spending time in a chamber containing either a novel mouse or a novel object, sociability is defined as a preference for spending time with a novel mouse. During both trials, locomotor activity (distance travelled (cm); velocity (cm/s) and time (s) spent in each chamber) were tracked using EthoVision (Noldus Information Technologies, Wageningen, The Netherlands; http://www.noldus.com/site/doc200403002).

Rotarod

The Rotarod is a method used to assess motor learning and coordination in mice (Wohr et al., 2013). Mice were tested at approximately PND 50 using the Rota-Rod 47600 device (Ugo Basile, Milan, Italy). Testing was run over two days and the rotarod was set to forward acceleration from 0-40 revolutions per minute throughout. On the first day, mice underwent three consecutive trials, 5 minutes in length followed by an hour break, then a further three trials of 5 minutes. This was then repeated the following day to assess motor learning. Latency to fall (s) was recorded, with higher latencies indicating better motor coordination and the increase in latency occurring throughout the two days being indicative of motor learning.

Adult Social Investigation

Social investigations of adult mice were assessed as described previously (Grayton et al., 2013). Mice were tested at approximately PND 65. Mice were transferred to a clean cage, identical to their normal home cage (Techniplast cage, 32cm x 16cm x 14cm) containing only sawdust at a height of 2 cm, 1 hour before testing to habituate. The testing area was dimly lit from below (10 lux). The tails of the conspecific mice were marked with a pen (Pentel, UK) so they can be identified in the recording. During testing, mice were transferred into the testing room in their 'new' home cage, and an age and sex-matched novel conspecific mouse was put into the cage with them. The mice were allowed to interact and recorded for 5 minutes. When aggression was observed for prolonged periods (>2 minutes), the trial was stopped, and the conspecific mouse was removed. Following testing, the conspecific was removed and taken back to its home cage, and the test mouse was taken out of the test room, enrichment added to the cage and returned to the housing room. Social behaviours initiated by the test mice were scored from the recordings by researchers who were blind to the genotypes of the

mice. The details of the measures scored can be seen in Table 3 (see Winslow, 2003 and Schneider et al., 1992).

Table 3. Items scored in the adulthood social investigation test. The number, latency and duration of each behaviour were recorded.

SOCIAL INVESTIGATION BEHAVIOURS		
Investigative behaviours	Social sniffing	Sniffing of the body above the shoulders
	Following	Behaviour where the test mouse moves in close proximity to the conspecific without making direct contact with the mouse
	Mutual circle	Partners are mutually sniffing each other's anogenital region, while moving in tight circles together with their reciprocal following movements
Affiliative behaviours	Social grooming	Mice groom each other
	Social rest	Test animal is being groomed by the conspecific
	Push under	Test animal pushes its own snout or the whole anterior part of its body under the conspecific's body, and rests for at least 3 s
Social interaction-soliciting items	Social inactive	Test animal is lying flat or standing still (eyes closed or open) while maintaining close physical contact with the conspecific
	Crawl under/over	Test animal crawls underneath/over the conspecific's body, crossing it transversely from one side to the other (only number recorded, as duration cannot be reliably estimated)
	Push past	Test animal passes between the wall of the cage and the body of the conspecific by pushing its own body through the narrow space available (only number recorded, as duration cannot be reliably estimated)
Aggression	Attacks	Biting opponent mouse

3-chamber Social Approach (Adult Conspecific Mice)

Individuals with ASD may display abnormal social cues (Chevallier et al., 2012). Based on this, we performed the 3-chamber social approach task again in the mice as young adults (approximately PND

72) following the protocol described in Section x but with the position of the test mouse and the conspecific mouse reversed. The aim was to see whether the conspecific mouse would spend less time with a knock-out test mouse under a wire cage than it would with a wild-type or heterozygote.

Marble Burying

Marble burying is a test used to evaluate repetitive behaviour in mouse models (Deacon, 2006; Thomas et al., 2009). Testing was performed in a dimly lit test room (10 lux) when mice were at approximately PND 80. For each mouse, a Techniplast cage (32cm x 16cm x 14cm) was filled with 5 cm sawdust and 12 marbles were placed equidistant from each other in a 3 x 4 array covering ¾ of the arena, while the remaining ¼ of the cage was left clear for the addition of the mouse. Upon addition of the mouse to the empty ¼ of the cage, a clear Perspex lid was used to cover the cage and contain the mouse while allowing recording to take place by an overhead camera. The test ran for 30 minutes, during which time the mouse could freely explore the cage and bury marbles. After 30 minutes, the mouse was removed and marbles that were buried up to at least ⅔ of their height were recorded. Additionally, the number of buried marbles was checked at 10 and 20 minutes to ensure that mice were not burying and un-burying marbles.

Olfactory Habituation/Dishabituation

Olfactory habituation is used to assess deficits in olfaction (Yang and Crawley, 2009). As social behaviour relies heavily on olfaction (Zou et al., 2015), this test is a necessary control for the interpretation of the social behaviour. Previously a buried cookie test showed that there were no olfactory deficits in *Nrxn1α* knock-out mice (Grayton et al., 2013), however a recent study showed that outcomes of the buried cookie test and olfactory habituation testing can differ given the former test can be influenced by the mouse's appetitive behaviour (Gusmão et al., 2012). Therefore, this test was performed on the second batch of test mice to ensure there are no olfactory deficits. This test was performed in batch 2 only, in which there were 45 mice (Males; 6 WT, 8 HET, 8 KO and Females; 8 WT, 7 HET, 8 KO). Animals were tested at approximately PND 85 in their home cage, which had been cleaned out 3 days prior to testing, with all enrichment removed and a fresh cage lid to minimise interfering odours. Following a 10 minute habituation the mouse was exposed to three odours in turn: water (control/no odour; 50µl), banana essence (non-social; 50µl, 1:100 dilution; Uncle Roy's, Moffat, UK) and urine collected from a novel, sex-matched conspecific (social, 25µl). Each odour was presented on a cotton-tipped wooden applicator 3 times and for a period of 2 minutes each time with an interval of roughly one minute while the next cotton bud was prepared. The total time (s) spent by the mouse sniffing each cotton bud during every trial was recorded. Habituation to an odour was

defined as a decrease in sniffing over consecutive presentation of the same odour, and dishabituation as an increase of sniffing when a new odour is presented.

2.2.5 Statistical Analysis

Data were analysed using GraphPad Prism version 7.00 for Windows (GraphPad Software, California, USA). The effects of genotype within each sex was analysed using one-way ANOVA or repeated measures ANOVA, as appropriate, followed by Tukey's multiple comparisons test. USV and motor behaviour analysis was performed using a mixed-model Analysis of Variance with repeated measures but, since no sex differences were detected, USV and motor behaviour data from males and females were combined. For developmental milestone testing, all data were analysed using non-parametric Kruskall-Wallis, since the data collected in this section is ordinal.

Results

Ultrasonic Vocalisations

Nrxn1α KO mice showed abnormal ultrasonic vocalisations

Although the ontogenetic profile of pups' ultrasonic vocalisations emitted from age PND 2 to 12 did not follow an inverted U-shape, there was a main effect of day ($F(4,436)= 3.148$, $p= 0.0144$). Starting from PND 4, both WT and KO pups rapidly increased the ultrasonic vocalisation emission with a peak at PND 12, while the profile of emission appeared quite flat in HET pups. Moreover, the ultrasonic calling rate varied across genotype ($F(2,109)= 6.485$, $p= 0.0022$). Figure 1A showed that KO mice emitted significantly less vocalisations than their WT and HET littermates upon separation from their mother and siblings.

Analysis of the USVs emitted across the first 12 postnatal days of age detected significant differences between WT, HET and KO pups in the mean call duration ($F(2,109)= 29.018$, $p< 0.0001$; see Figure 1B). Post hoc comparisons performed on the two-way interaction genotype x postnatal day ($F(8,436)= 3.806$, $p= 0.0002$) indicated that KO pups emitted shorter calls than WT and HET pups at every PND assessed ($p< 0.01$), except on PND 8 when KO showed different call duration only compared to HET pups. On PND 2, mean duration of vocalisations also differed across WT and HET pups ($p< 0.01$). Peak of the frequency and amplitude did not differ between genotypes (respectively, $F(2,109)= 0.191$, $p= 0.8262$; $F(2,109)= 0.584$, $p= 0.5596$, data not shown).

Analysis of neonatal vocal emission did not vary when accounting for body weight and body temperature through a linear mixed model.

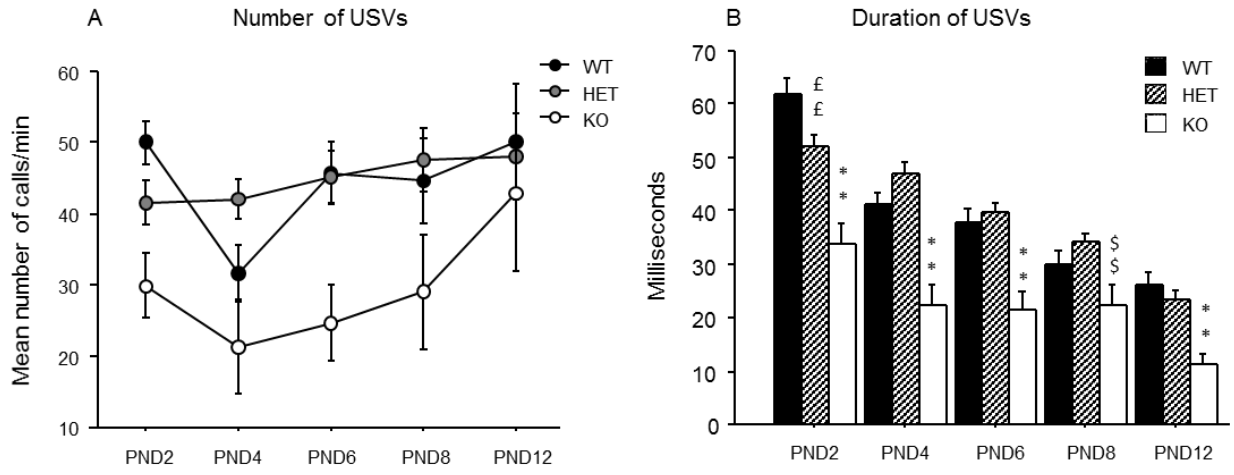

Figure 1. Ultrasonic vocalisations (USVs) in *Nrxn1α* pups. Mean (± SEM) number (A) and duration (B) of ultrasonic vocalisations on PND 2, 4, 6, 8 and 12 in response to social separation during a three-minute-session. Data derived from 33 WT (15 males and 18 females), 58 HET (26 males and 32 female), 21 KO (11 males and 10 females). Levels of significance indicated by ** p <0.01 KO vs HET and WT; by ££ p <0.01 HET vs WT; $$ p <0.01 KO vs HET.

Specific pattern of sonographic structure among *Nrxn1α* mice

WT, HET and KO pups emitted a wide spectrum of call categories. Production of calls within each call category is shown in the pie chart (Figure 2). On PND 2, WT pups (see first column of the pie chart) mainly emitted *complex*, *two-components* and *downward* USVs, along with a low prevalence of *chevron*, *short*, *composite* and *flat* call categories. On PND 4, WT pups showed a decrease in the emission of the *complex* calls in favor of the *two components* and *short* subtypes. On PND 6, as well as on PND 8, a reduction of *complex* calls and increase of *short* calls appeared more pronounced compared to PND 2 and to PND 4. Moreover, on PND 6 WT pups started to emit calls belonging to *frequency steps* categories. On PND 12, the general vocal repertoire was mainly defined by the concomitant presence of three types of calls: *complex* (29%), *two-components* (15%) and *short* (44%).

HET pups (see second column of the pie chart) exhibited a differential vocal production in comparison to the other two genotypes: it could be defined as an intermediate phenotype between WT and KO pups. Indeed, HET mice already on PND 2 emitted a reduced proportion of *complex* and an increased proportion of *downward* call subtypes compared to WT pups at the same age. However, on PND 4 and PND 6, HET vocal profile is characterized by the *complex, two-components* and *short* calls, in a more similar way to WT than KO vocal profile. On PND 12 the emission of *short* calls by HET pups reached

the maximum in comparison to other postnatal days (51% of emission), determining a reduced production in *complex* and *two-components* categories.

Analysis of the KO vocal repertoire (see third column of pie chart) indicates that they produced mainly three call type calls (*complex*, *two-components* and *short*), as WT and HET mice, but with substantial differences in the frequency for each ultrasonic subtype. Over the first two postnatal weeks, KO pups emitted a decreased number of *complex* and *two components* calls and an increased number of *short* calls, if compared to control and HET pups. Already on PND 6, the emission of *short* calls reached and exceeded the fifty percent of vocal emission (62%) and represented almost the full pie chart of KO production on PND 12 (81%). This data indicated that KO pups persisted in emitting primarily the *short* calls from PND4 to PND 12.

Classification of ultrasonic vocalisations into distinct call categories within *Nrxn1α* KO mice

Figure 3 illustrates the genotype-dependent variation in frequency of calls at PND 2 to PND 12. Analysis of the frequency of call types at PND 2 revealed that both HET and KO pups emitted fewer *complex* calls than WT, while KO pups produced less *two components* calls compared to HET. A significantly higher number of *short* calls was emitted by KO compared to WT and HET pups (after post hoc comparison performed on genotype x calls subtype interaction: $F(16,872)= 4.560$, $p< 0.0001$).

As illustrated in Figure 3B, a genotype-dependent effect was found at PND 4 ($F(2,109)= 12.294$, $p< 0.0001$), with KO emitting significantly less *complex* and *two-components* calls and more *short* calls than the other two genotypes (genotype x calls subtype interaction: $F(16,872)= 8.352$, $p< 0.0001$).

At PND 6, KO pups produced significantly less *two-components* and more *short* calls than WT and HET (genotype: $F(2,109)= 6.085$, $p< 0.01$; genotype x calls subtype interaction: $F(16,872)= 11.764$, $p< 0.0001$). At this specific time point, the ultrasonic emission differed within the *downward* call subtype with KO pups emitting less calls of this type in comparison to WT.

At PND 8, similar to the vocal profile analyzed at PND4, KO pups emitted less *complex* calls than HETs and less *two-components* and more *short* calls than WTs and HETs (genotype: $F(2,109)= 6.406$, $p< 0.001$; genotype x calls subtype interaction: $F(16,872)= 4.442$, $p< 0.0001$). Over the last postnatal day of recording (PND 12), KO emitted less *complex* and more *short* calls than WTs and HETs (genotype: $F(2,109)= 4.658$, $p< 0.05$; genotype x calls subtype interaction: $F(16,872)= 5.672$, $p < 0.0001$). Moreover, KO emitted less *two-components* call subtypes than WT pups.

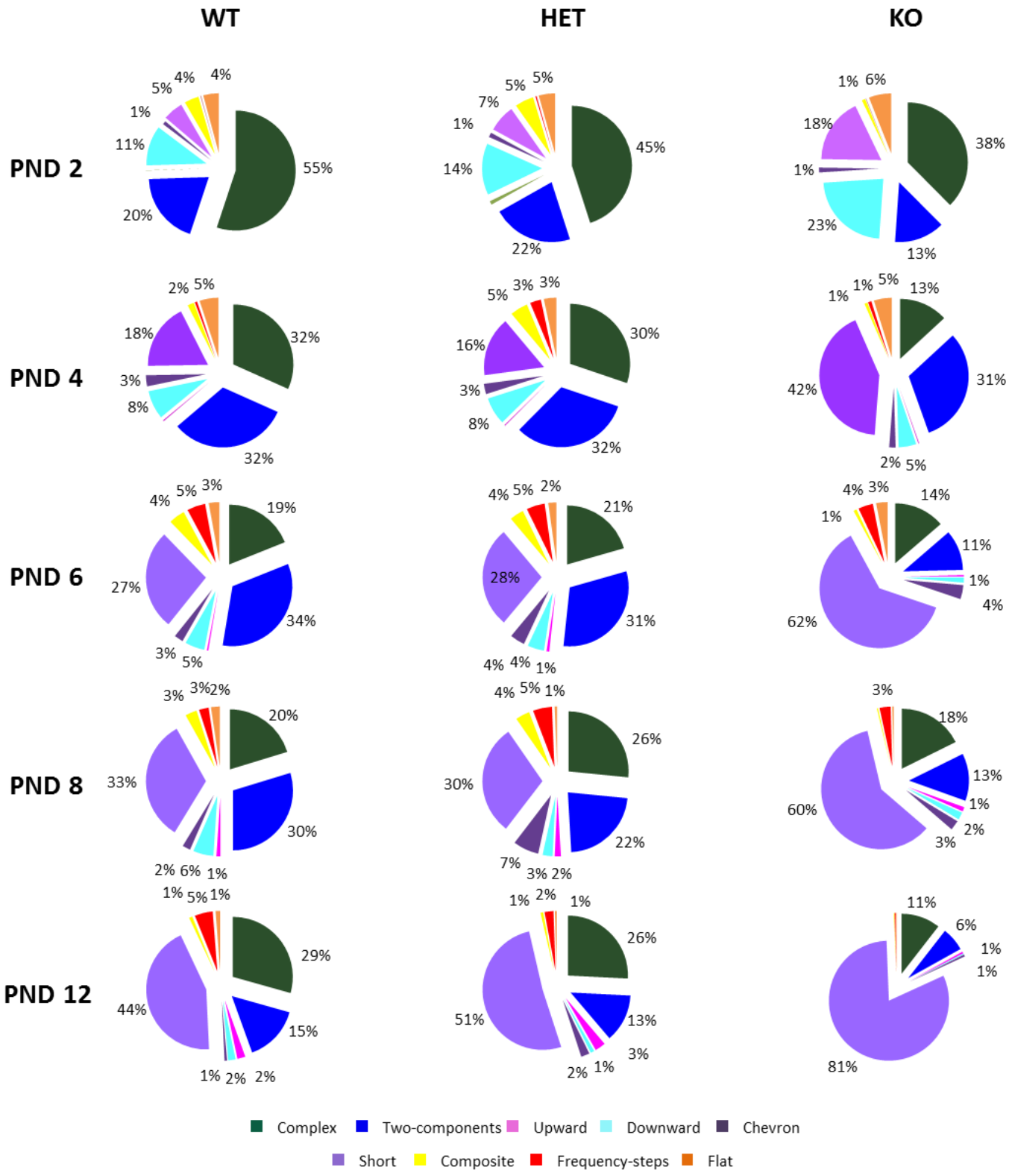

Figure 2. Pie charts showing the percentages of the different call categories for *Nrxn1α* pups during the five days of testing (PND 2, 4, 6, 8, 12). Percentages were calculated for each genotype as the number of calls in each category for each mouse/total number of calls analysed for each mouse.

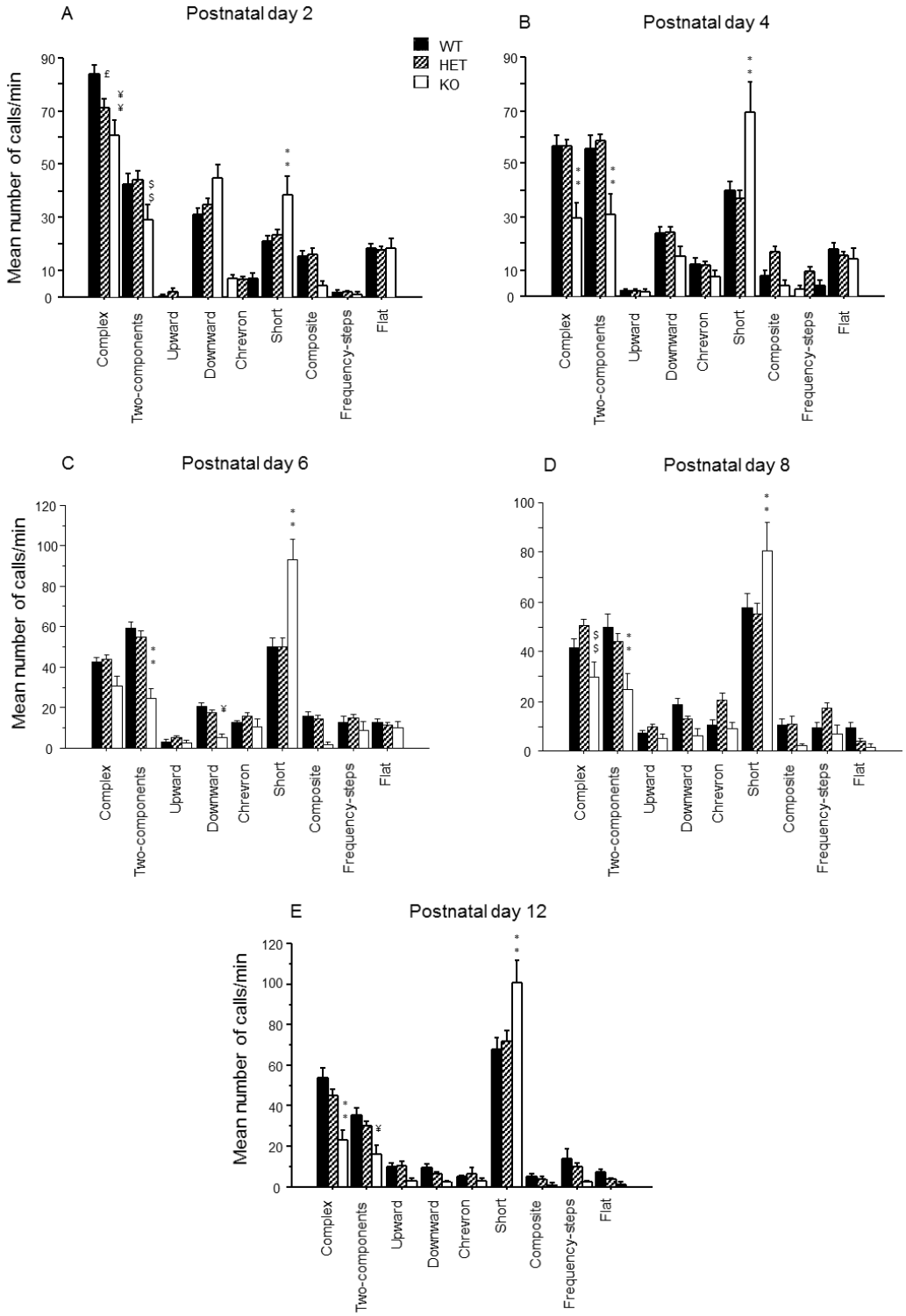

Figure 3. Ultrasonic vocalisations in *Nrxn1α* mice by call category. Mean (± SEM) number of ultrasonic vocalisations at PND 2 (A); at PND4 (B); at PND 6 (C); at PND 8 (D); at PND 12 (E). Data derived from 33 WT (15 males and 18 females), 58 HET (26 males and 32 female), 21 KO (11 males and 10 females). After post hoc, levels of significance indicated by ** p <0.01 KO vs HET and WT; $$ p <0.01 KO vs HET; ¥ p <0.05 or ¥¥ p <0.01 KO vs WT; £ p <0.05 HET vs WT.

Differences in body temperature, body weight and righting reflex in *Nrxn1α* mice

Pups' body temperature, body weight and righting reflex (measured as latency to turn back onto all four paws when placed on the back) were measured each day immediately after ultrasonic vocalisation recording. Body temperatures of mutant pups differed from control littermates across the five days of testing (genotype: $F(2,109)= 3.419$, $p< 0.0363$; day: $F(4,436)= 275.255$, $p< 0.0001$; interaction: genotype x day: $F(8,436)= 1.640$, ns), with KO mutants showing a decreased temperature when compared to controls (Figure 4A).

Body weight differed significantly between genotypes ($F(2,109) = 9.385$, $p< 0.0002$) across the five days of testing ($F(4,436) = 4785.520$, $p< 0001$). Post hoc comparisons performed on the two way interaction genotype x day ($F(8,436)= 13.589$, $p< 0.0001$) showed that KO pups were lighter than HET at PND 2 and HET and WT pups from PND 4 through PND 12 (Figure 4B).

As expected, significant differences were found in righting reflex latencies over time (day: $F(4,436)= 216.029$, $p< 0.0001$), with all three genotypes reaching the full development of the reflex on PND 12. However, a significant deviation from normative motor development was present in KO pups. Post hoc comparisons performed on the interaction genotype x day ($F(8,436)= 2.313$, $p< 0.0195$) indicated that KO pups spent more time to turn their body on PND 4 than littermate controls and HETS (Figure 4C).

***Nrxn1α* deletion was associated with altered spontaneous motor behaviours**

Several abnormalities in the acquisition of the spontaneous motor responses were also detected during the first two postnatal weeks. From PND 2 to 6, when pups are generally unable to move around properly, they pivot with the forelegs. Pivoting duration rapidly increased across the days of observation as demonstrated by the main effect of the age ($F(2,218)= 14.019$; $p< 0.001$). Statistical analysis also showed a main effect of the genotype for pivoting duration ($F(2,109)= 10.838$; $p< 0.001$) with KO pups spending more time in pivoting than HET and WT littermates (Figure 5A). A similar motor alteration was observed for duration of wall climbing, measured from PND 6 to 12 when pups start to move around using all four legs (Figure 5B). The time spent in performing wall climbing increased from PND 6 to 12 ($F(2,218)= 27,883$; $p< 0.001$) and a genotype difference was also detected ($F(2,109)= 3,703$; $p< 0.0275$). A hyperactive profile of *Nrxn1α* KO pups was confirmed by the analysis of both duration and frequency in locomotion (respectively, Figures 5C and 5D). The locomotor activity rapidly increased across the days of observation (duration: $F(2,218)= 96.050$; $p< 0.0001$ and frequency $F(2,218)= 139.088$; $p< 0.0001$). Post hoc comparisons performed on the two-way interaction genotype x days (duration: $F(4,218)= 6.890$; $p< 0.0001$; frequency $F(4,218)= 3.837$; $p< 0.0049$) indicated that KO

mice were more active than HET and WT on PND 12. To confirm this, ANOVA showed a main effect of the genotype in the number of immobility events ($F(2,109)= 10,128$; $p< 0.0001$) and a significant interaction between genotype x days ($F(8,436)= 2,223$; $p< 0.0249$). Post hoc comparisons reported that KO pups performed a reduced number of immobility events when compared to HET and WT pups on PND12 (Figure 5E).

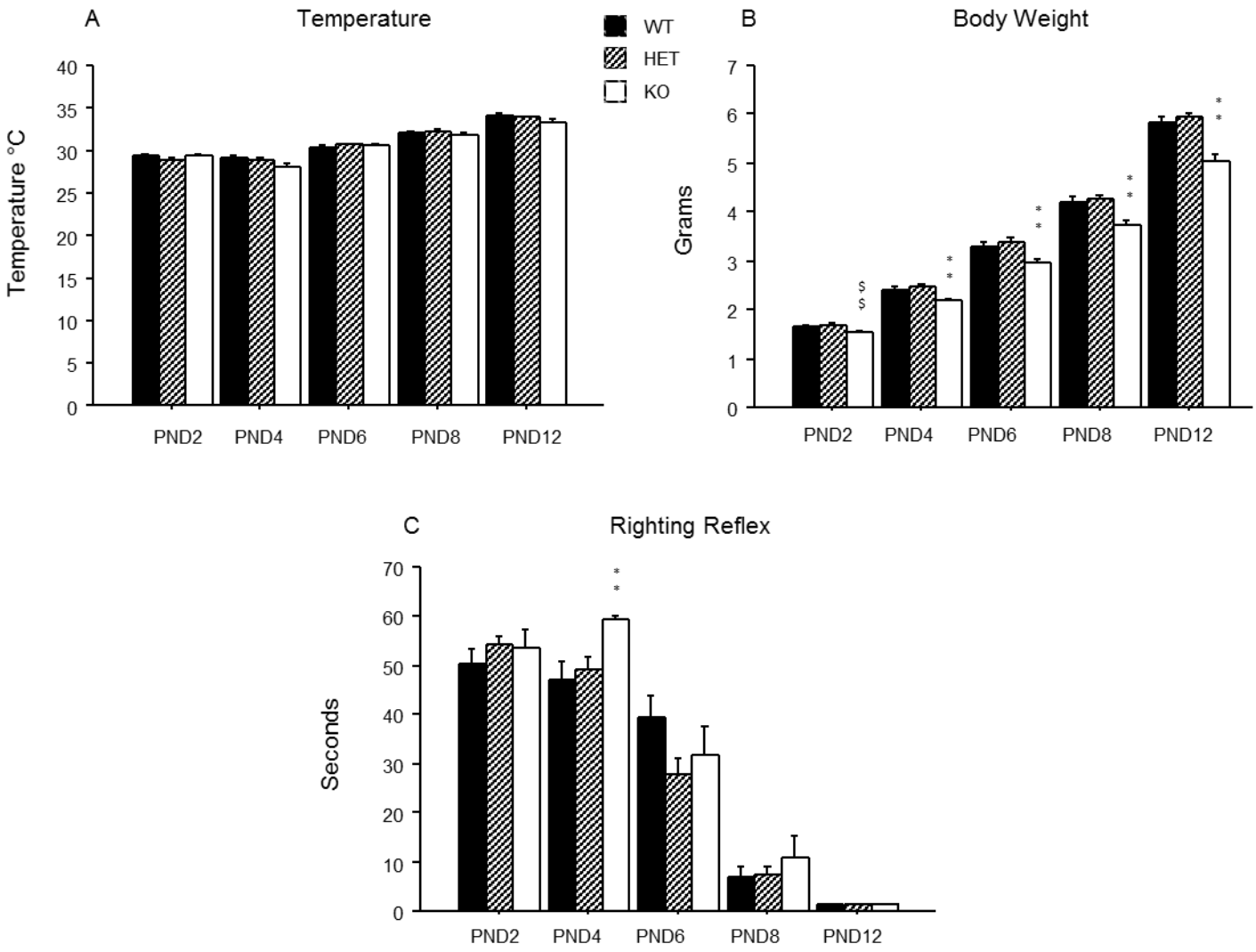

Figure 4. Mean (± SEM) body temperature (A) body weight (B), righting reflex latencies (C) in *Nrxn1α* mouse pups that were tested for ultrasonic vocalisations. Data derived from 33 WT (15 males and 18 females), 58 HET (26 males and 32 female), 21 KO (11 males and 10 females). Data are expressed as

means ± SEM. After post hoc, levels of significance indicated by ** p <0.01 KO vs HET and WT; $$ p <0.01 KO vs HET.

However, when a fine motor coordination and equilibrium were required, *Nrxn1α* KO pups exhibited a less active profile. KO mice exhibited a lower number of face washing episodes than the other two genotypes at PND 12 ($F(2,109)= 3,287$; $p< 0.0411$), suggesting a clear motor deficit in KO pups in the ability to standing up on their hindlimbs and wash their faces with the forelimbs (Figure 5F). Moreover, KO pups showed a lower number of head shaking and head rising episodes than HET and WT pups. ANOVA detected a main effect of genotype on frequency of head shaking ($F(2,109)= 2.912$; $p= 0.0586$) and of head rising ($F(2,109)= 3.871$; $p< 0.0238$) with KO pups performing less head spontaneous behaviours than WT littermates at all days of testing (Figures 5G and H). No differences between genotypes were observed for curling, side or circling behaviours (data not shown).

Developmental Milestones

Body weight was significantly lower in male *Nrxn1α* KO mice, but not female *Nrxn1α* KO mice

Male and female body weight was measured at PND 3, 5, 7, 9, 11, 13 and 15. In males, KO mice had significantly lower body weight at PND 9: $F(2, 36)= 8.43$, $p= 0.001$, PND 11: $F(2, 36)= 6.33$, $p= 0.004$, PND 13: $F(2, 36)= 6.66$, $p= 0.004$ and PND 15: $F(2, 36)= 6.15$, $p= 0.005$. Female body weight did not differ significantly between genotypes (Figure S1). In adulthood, the body weight of *Nrxn1α* KO mice is significantly but only slightly reduced in both males and females (genotype factor: $F(2,64)= 14.25$, $p= 0.001$, Figure S2).

Body length was significantly lower in male *Nrxn1α* KO mice, but not female *Nrxn1α* KO mice

Male and female body and tail length were measured at PND 3, 7 and 11 (Figures S3, panels A-D). Male body length was found to be significantly shorter in KO mice than their HET and WT littermates at PND 11 only (Body length: $F(2, 36)= 4.42$, $p= 0.02$). However, there was not a significant difference between male tail length and in females no changes to body length or tail length were observed.

Mice with *Nrxn1α* deletion showed delays in vertical screen

The vertical screen grasp test measures the ability of the pup to hold on to a wire mesh screen when pulled gently by the tail across the screen in a vertical position (Crawley, 2007). Vertical screen score was measured in mice at PND 5, 7, 9, 11, 13 and 15 (Figure S3, panels E-F). Both male and female *Nrxn1α* KO mice showed significant delays in grasping the vertical screen on the final day of testing, PND 15, in comparison to WT and HET littermates (Male mice: $H (2, n=39) = 9.29$, $p= 0.0096$, female mice: $H (2, 43) = 10.27$, $p= 0.0059$).

Male mice with *Nrxn1α* deletion showed delays in negative geotaxis

Negative geotaxis involves placing the pup on an inclined plane with the pup's head facing downwards. The ability of the pup to change orientation to face up the plane was assessed (Crawley, 2007).

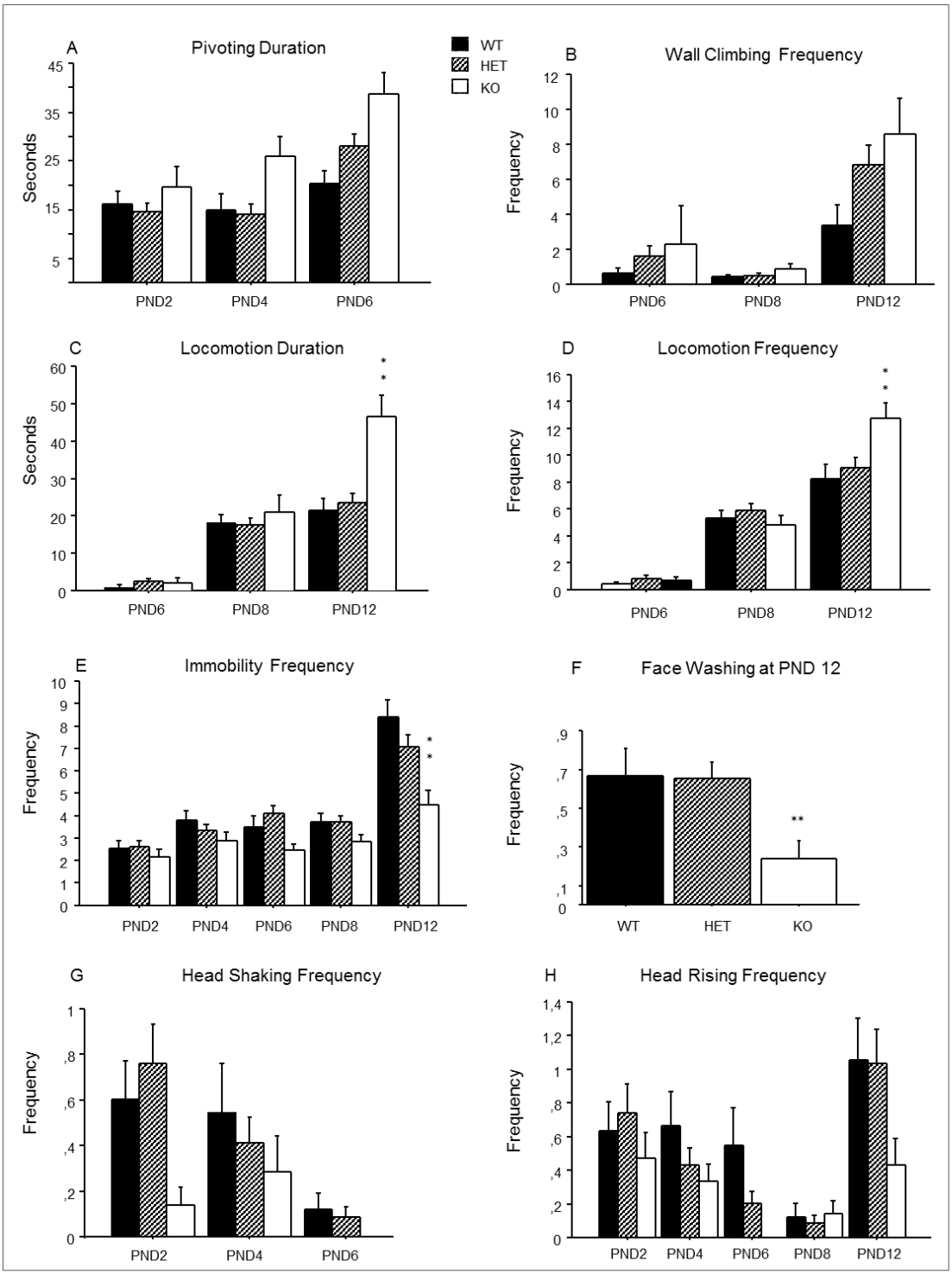

Figure 5. Mean (± SEM) number and/or duration of spontaneous motor behaviours shown by Nrxn1α pups on PND 2, 4, 6, 8 and 12 during a 3-min session: (A) Pivoting, (B) Wall Climbing, (C-D) Locomotion, (E) Immobility, (F) Face Washing, (G) Head Shaking, (H) Head Rising. Data derived from

33 WT (15 males and 18 females), 58 HET (26 males and 32 female), 21 KO (11 males and 10 females). Data are expressed as means ± SEM. Levels of significance indicated by ** p <0.01 KO vs HET and WT.

Testing was carried out on PND 9, 11, 13 and 15 (Figure S3, panels G-H). Male *Nrxn1α* KO mice showed severe delays in orientating themselves upwards at PND 13 ($H(2, n=39)= 8.04$, $p= 0.02$) and PND 15 ($H(2, n=39)= 20.10$, $p= <0.0001$) with most pups failing to hold themselves on the wire mesh during the trial on each day. Female *Nrxn1α* KO did not show delays in completing this test.

Nrxn1α KO mice displayed delayed ear canal opening

Ear canal opening was measured at PND 13 and 15; however, the first opening was not observed until PND 15 (Figure S4). Both male and female KO mice showed a significant delay in ear opening, with almost all WT and HET pups displaying full ear canal opening by PND 15 while less than half of KO littermates reached the milestone at this stage (Males: $H(2, n=39)=19.85$, $p= <0.0001$ and females: $H(2, n=43)= 12.87$, $p= 0.002$).

Nrxn1α KO mice showed a delay in cliff avoidance

Cliff avoidance was used as a measure of gross visual ability, during which a pup was placed on an apparatus with an opaque side and a clear Perspex side resembling a cliff-like drop. The pup must turn away from danger and crawl onto the opaque part of the apparatus (Fox, 1965). This developmental milestone was measured at PND 15 only, since the test requires pups' eyes to be open (Figure S5). Male *Nrxn1α* KO mice showed significant delays in cliff avoidance, with most KO pups running off the apparatus immediately ($H(2, n=39)= 20.13$, $p= <0.0001$). Female *Nrxn1α* KO mice did not show significant delays, however there was a strong trend towards a delay in cliff avoidance compared to WT littermates ($H(2, n=43$, $p= 0.06$).

Nrxn1α deletion was not associated with reduced grasping reflex, surface righting, fur appearance, incisor eruption, horizontal screen, quadrupled walking, auditory startle, tactile startle or eye opening (data not shown).

Deletion of *Nrxn1α* did not cause olfactory deficits

The *Nrxn1α* deletion did not result in olfactory deficits in mice (Figure S6). Normal habituation/ dishabituation profiles were observed in all groups characterised by a decrease in sniffing latencies between the first and last exposure to particular odour, followed by reinstatement of sniffing when a new odour was presented and marked increase in sniffing durations for the social condition. This

confirms that social data was not confounded by differences in olfaction between WT, HET and KO mice of either sex.

Male juvenile *Nrxn1α* HET and KO mice showed altered social investigative behaviour

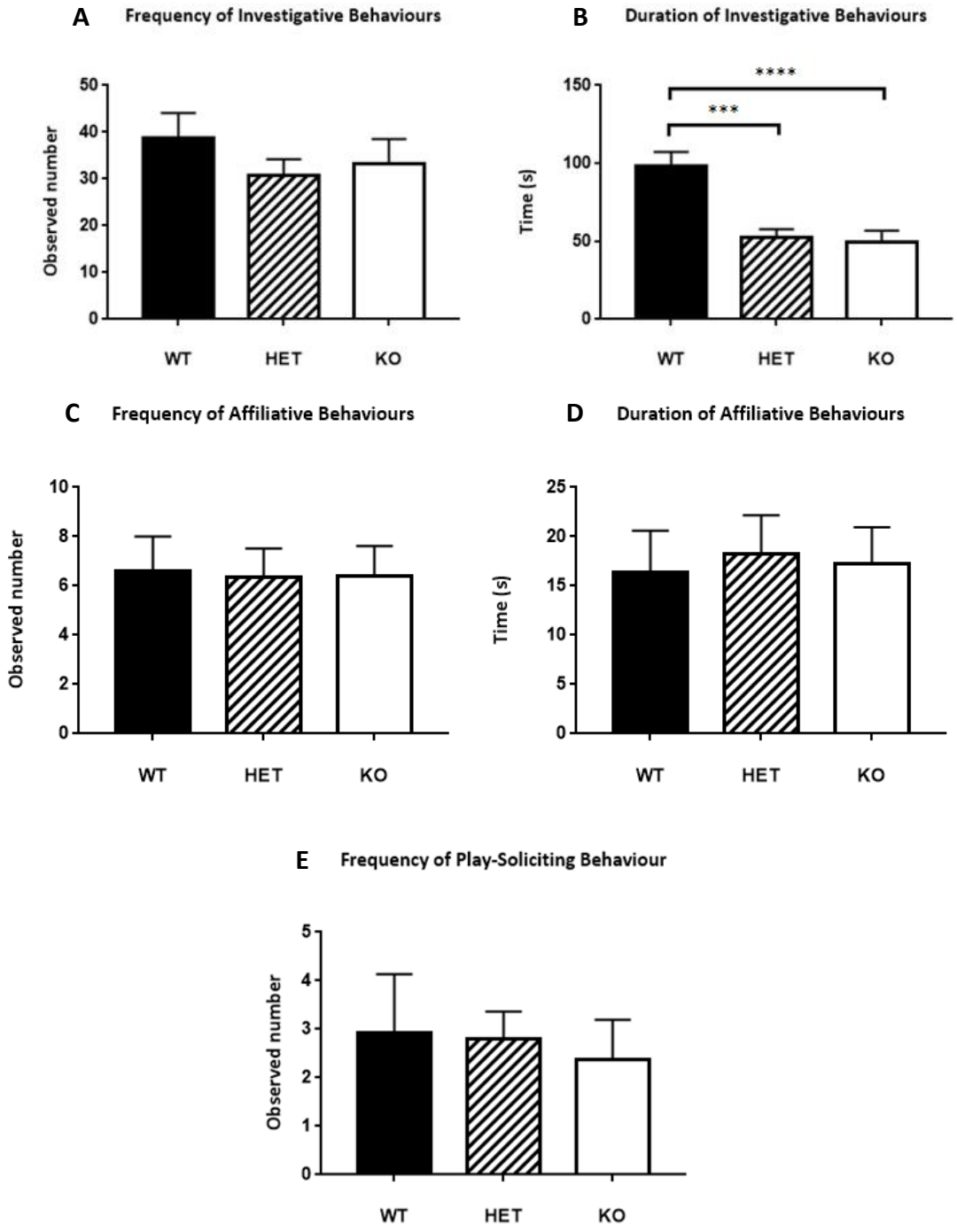

Figure 6. Profile of investigative, affiliative and play-soliciting behaviours in juvenile male *Nrxn1α* mice. Mean (± SEM) number (A) and duration (B) of investigative behaviours; number (C) and duration (D) of affiliative behaviours and number of play-soliciting behaviours (E). Data derived from 12 WT, 19 HET and 19 KO males. Levels of significance indicated by *** $p < 0.001$ and **** $p < 0.0001$.

For analysis of juvenile social behaviour, scored behaviours were grouped into investigative, affiliative and play-soliciting behaviours as listed in Table 3. A strong sex effect was present between males and females, therefore the effects of genotype within each sex were analysed separately by one-way ANOVA. In males, no significant differences were seen in the frequency of investigative behaviours, however both *Nrxn1α* HET and KO mice spent significantly less time engaging in investigative behaviours than their WT littermates ($F(2, 47)= 13.16$, $p= <0.0001$, see Figure 6). When individual investigative behaviours were analysed, the social sniffing behaviour was significantly different (sniffing duration, $F(2, 47)= 7.06$, $p= 0.002$), while the following and mutual circle behaviours were not (Figure 7). This was the main investigative behaviour that was affected in males. Affiliative behaviours and play-soliciting behaviours (Figure 6) were unaffected by *Nrxn1α* deletion. In females, no significant differences were observed in investigative, affiliative of play-soliciting behaviours between genotypes (Figure 8). These findings suggest the *Nrxn1α* deletion does not cause social abnormalities in females during juvenile play testing.

Male adult *Nrxn1α* HET and KO mice showed increased aggression, decreased affiliative behaviours (in KO males only)

For analysis of adult social behaviour, scored behaviours were grouped into investigative, affiliative, social interaction-soliciting and aggressive behaviours as listed in Table 3. A strong sex effect was present between males and females, therefore the effects of genotype within each sex were analysed separately by one-way ANOVA. Male *Nrxn1α* KO mice spent significantly less time engaging in investigative behaviours than control littermates ($F(2, 48)= 7.42$, $p= 0.002$, Figure 9). When individual behaviours were analysed, duration of sniffing behaviour ($F(2, 48)= 7.06$, $p= 0.002$) was the only significantly different investigative behaviour as was seen in the juvenile data, while following and mutual circle behaviours were not significant (Figure 10). The frequency and duration of affiliative behaviours was also lower in male KO mice and behaviours reached significance when summed together ($F(2,48)= 5.25$, $p= 0.009$ and $F(2, 48)= 6.03$, $p= 0.005$ respectively, Figure 9). In addition, HET and KO mice also showed greatly increased levels of aggression, compared to WTs (frequency of attack, $F(2, 48)= 4.99$, $p= 0.01$; duration of attack, $F(2, 48)= 7.48$, $p= 0.002$ and latency of attack, $F(2, 48)= 14.3$, $p= <0.0001$, see Figure 11). All other behaviours did not differ between genotypes. In females, a deletion in *Nrxn1α* was not associated with alterations in investigative, affiliative or social investigative behaviours (Figure 12). Aggressive behaviours were only observed in 2 out of 14 KO female mice so it is unlikely to be a robust genotype effect.

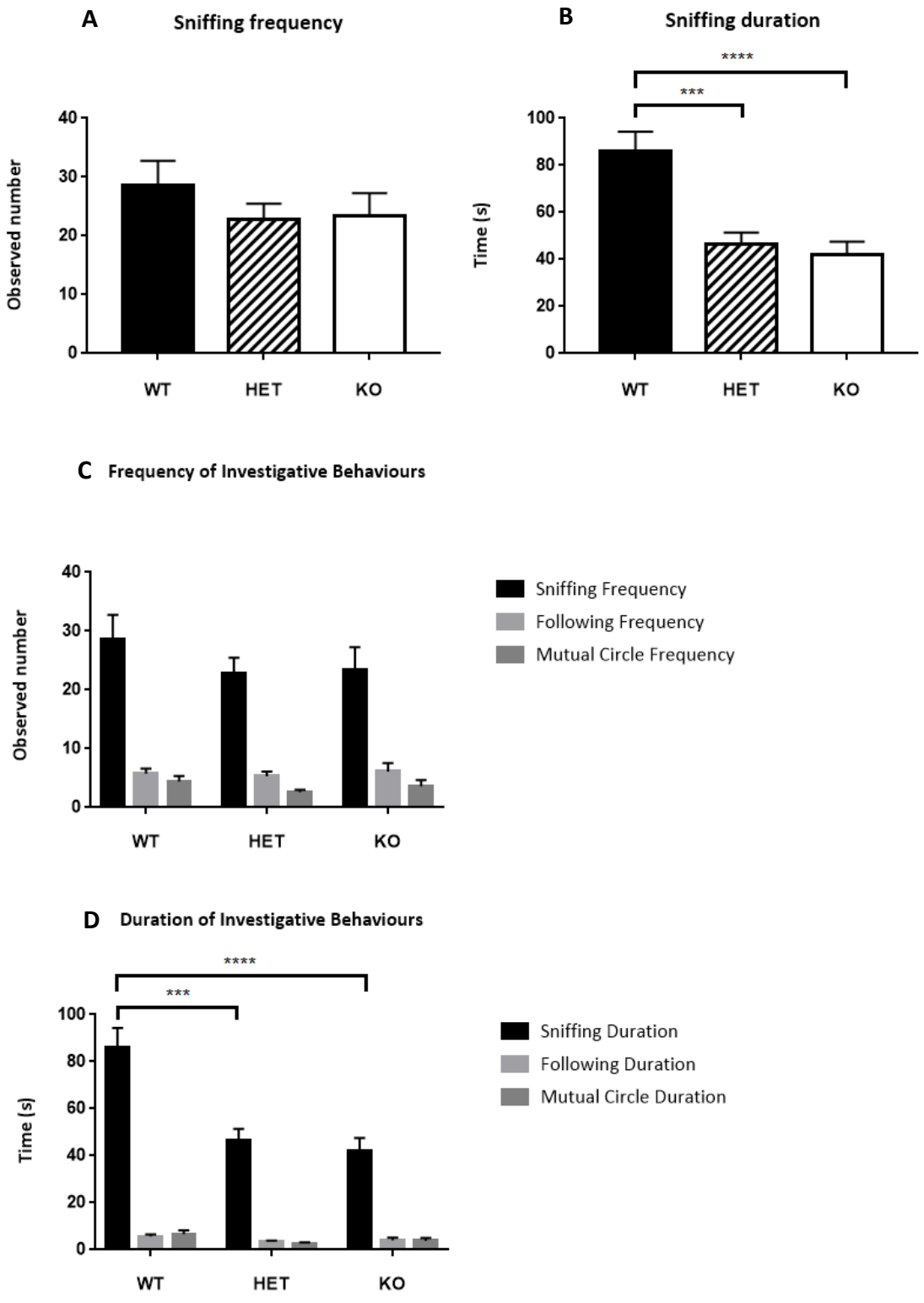

Figure 7. Individual investigative behaviours in juvenile male *Nrxn1α* mice. Mean (± SEM) number (A) and duration (B) of sniffing behaviour. Profile for number of individual investigative behaviours (C) and duration of individual investigative behaviours (D) show that social sniffing duration was the most affected behaviour. Data derived from 12 WT, 19 HET and 19 KO males. Levels of significance indicated by *** $p < 0.001$ and **** $p < 0.0001$, compared to WT.

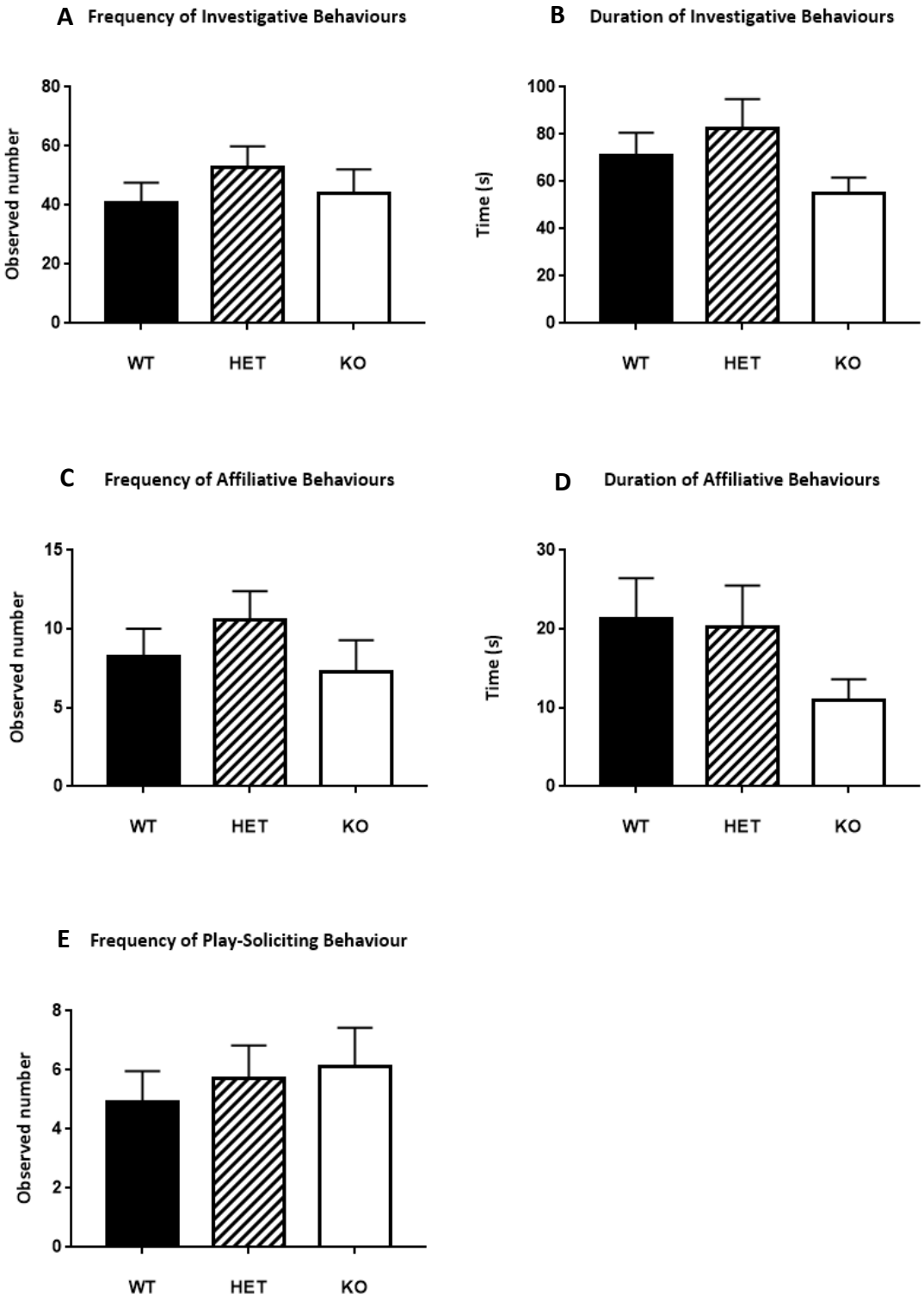

Figure 8. Profile of investigative, affiliative and play-soliciting behaviours in juvenile female *Nrxn1α* mice. Mean (± SEM) number (A) and duration (B) of investigative behaviours; number (C) and duration (D) of affiliative behaviours and number of play-soliciting behaviours (E). Data derived from 16 WT, 18 HET and 15 KO females.

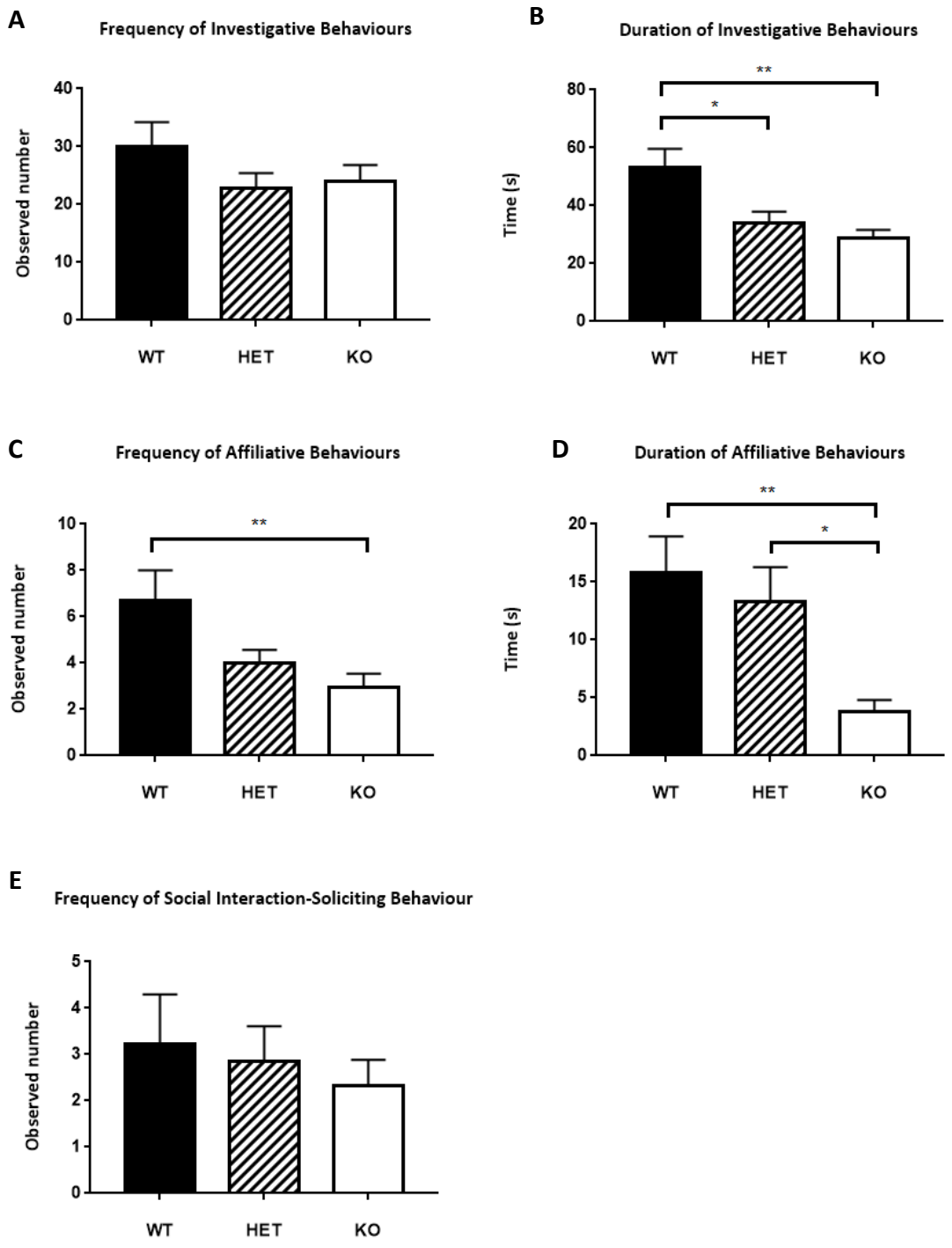

Figure 9. Profile of investigative, affiliative and social interaction-soliciting behaviours in adult male *Nrxn1α* mice. Mean (± SEM) number of investigative behaviours (A) did not differ between groups. Mean (± SEM) duration of investigative behaviours (B), number (C) and duration (D) of affiliative behaviours were reduced in KO mice. Number (E) and duration (F) of social interaction-soliciting behaviours were unchanged between groups. Data derived from 13 WT, 20 HET and 18 KO males. Levels of significance indicated by * $p < 0.05$ and ** $p < 0.01$.

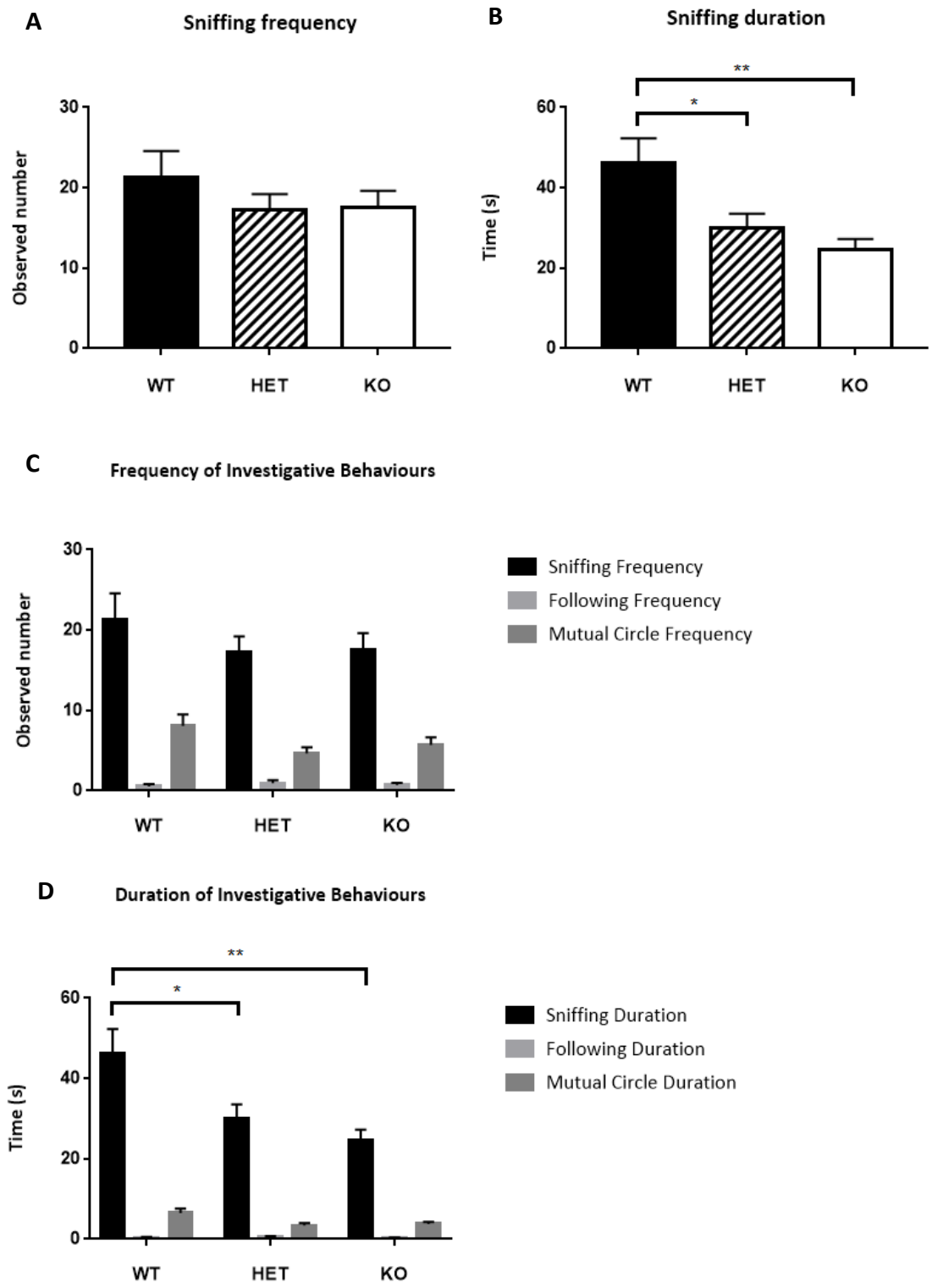

Figure 10. Individual investigative behaviours in adult male *Nrxn1α* mice. Mean (± SEM) number (A) and duration (B) of sniffing behaviour. Profile for number of individual investigative behaviours (C) and duration of individual investigative behaviours (D). Data derived from 13 WT, 20 HET and 18 KO males. Levels of significance indicated by * $p < 0.05$ and ** $p < 0.01$, compared to WT.

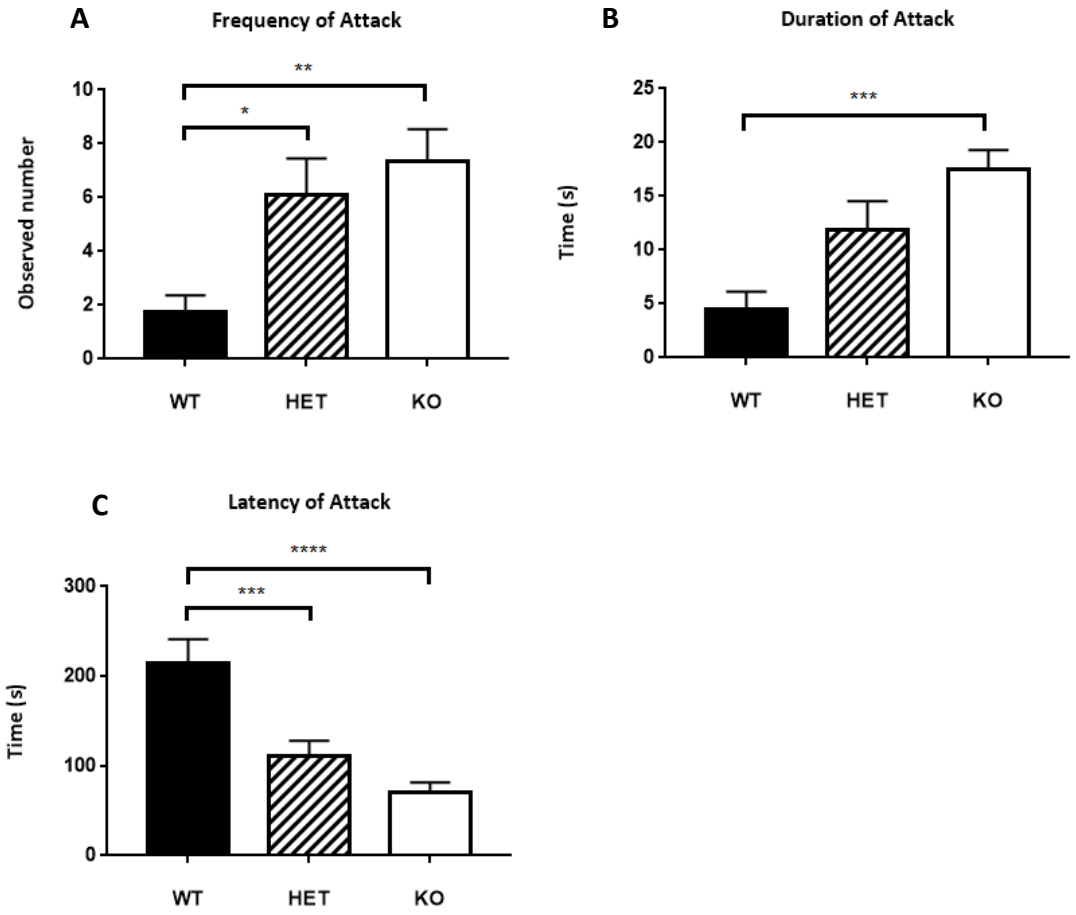

Figure 11. Profile of aggressive behaviours in adult male *Nrxn1α* mice. Mean (± SEM) number (A), duration (B), and latency (C) of attack. Data derived from 13 WT, 20 HET and 18 KO males. Levels of significance indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

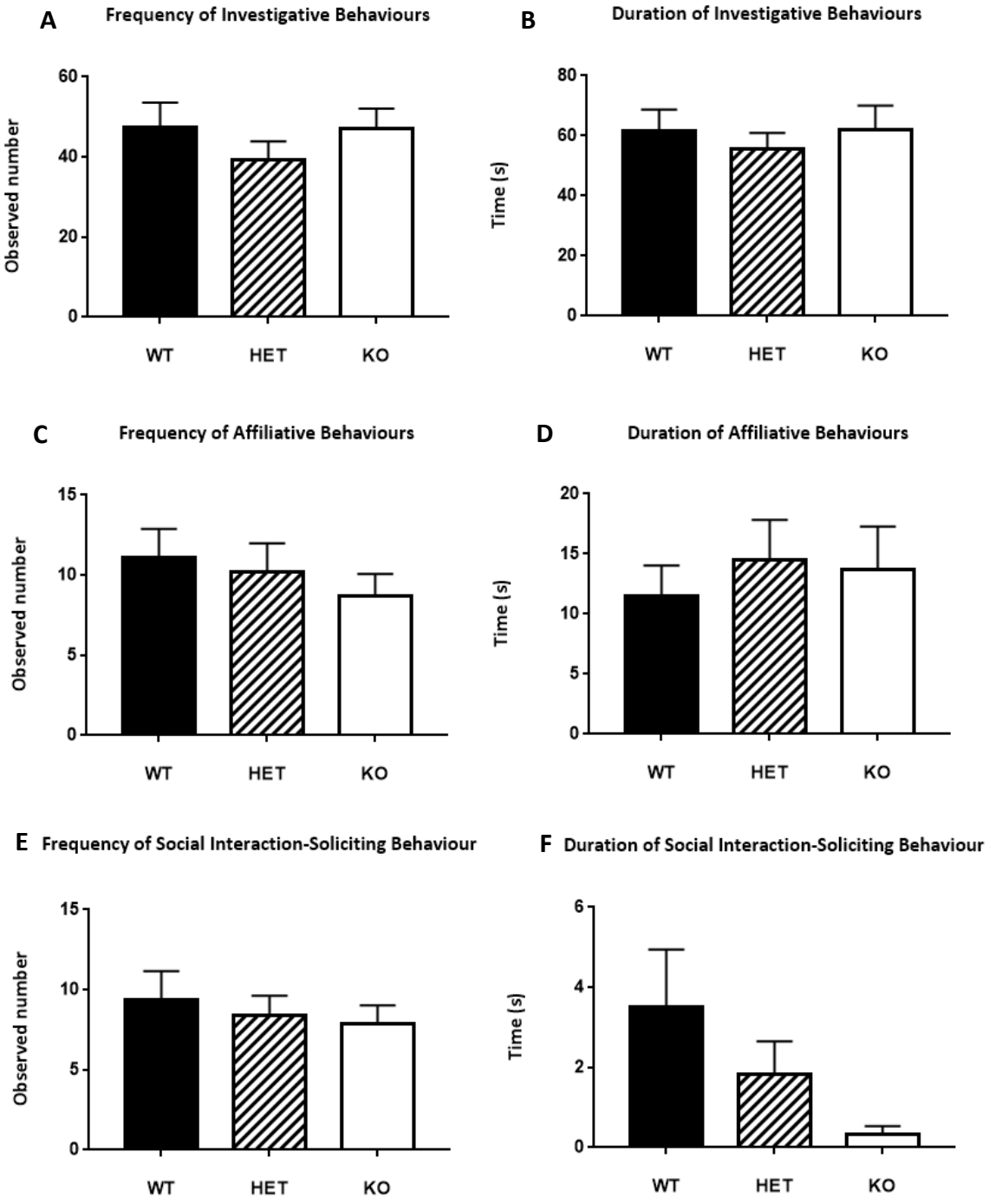

Figure 12. Profile of investigative, affiliative and social interaction-soliciting behaviours in adult female *Nrxn1α* mice. Mean (± SEM) number (A) and duration (B) of investigative behaviours, number (C) and duration (D) of affiliative behaviours and number (E) and duration (F) of social interaction-soliciting behaviours. No differences were observed between groups of mice. Data derived from 16 WT, 19 HET and 14 KO females.

Nrxn1α deletion did not result in social abnormalities in the juvenile three chamber social approach task

During Trial 1, the mouse was allowed to freely explore the three chambers of the apparatus. Analyses were performed to ensure there was no preference for either side of the chamber that could confound the results of Trial 2 (Figure S7). One sample t-tests confirmed that there was not a significant preference for time spent in the left versus the right chamber for any of the groups of mice. In addition, their movement was tracked, and the distance moved and velocity in each part of the apparatus recorded. Both male and female KO mice moved a significantly shorter total distance during Trial 1 ($F(2, 48)= 18.12$, $p= <0.0001$ and $F(2,46)= 18.72$, $p= <0.0001$ respectively, see Figure S8). For total velocity, both male KO mice ($F(2, 48)= 12.13$, $p= <0.0001$) and female KO mice ($F(2, 46)= 18.78$, $p= <0.0001$) showed significantly lower velocities than their HET and WT littermates (Figure S8).

During Trial 2, a novel mouse (social cue) and a novel object (non-social cue) were placed in the left and right chambers and the time spent in each chamber was tracked and recorded. Analyses were performed using the % time spent in the novel mouse chamber measure. A strong sex effect was present between males and females, therefore the effects of genotype within each sex were analysed separately. In males, no preference was seen for the social cue in WT, HET or KO mice (Figure S9). The premise of this task is that control (WT) mice are expected to show a preference for the social cue (Yang et al., 2011) and this suggests that the test has not worked in juvenile male mice and cannot be interpreted. In females, a preference for the social cue was seen in mice ($F(2, 46)= 3.31$, $p= 0.05$), suggesting that the protocol worked in juvenile female mice but there was no significant effect of genotype (Figure S9).

Conspecifics spent more time with adult female _Nrxn1α_ KO mice than WT and HET littermates in the modified three chamber social approach task

This version of the 3-chamber social approach task used conspecifics in the place of test mice, and test mice (WT, HET and KO) in the place of “novel mouse”. As expected, during Trial 1 no differences in total distance moved or total velocity was seen between conspecific mice and no preference for either side of the chamber was seen. The male adult conspecific mice showed a preference for a social cue, meaning that the task worked (Figure S10). However, the conspecific mice did not show any preference for spending time with WT, HET or KO male mice. This suggests that KO mice do not give off abnormal social cues, causing conspecifics to avoid them. However, in females, the conspecific mice show a clear preference for the female KO mice ($F(2,46)= 5.50$, $p= 0.007$) and appear to be less interested in the WT females (Figure S10). There was not a preference for WT social cue over a novel object cue, suggesting that the protocol was not that robust and data from the female mice should be interpreted with caution.

***Nrnx1α* deletion did not cause deficits in motor learning and co-ordination**

Mice were assessed for motor learning and co-ordination using the rotarod task. Each mouse underwent three trials per session with two sessions per day, for two days. Although there was evidence of motor learning between day 1 and 2 (within factor: $F(3, 144)= 9.32$, $p= 0.01$ for males and $F(3,138)= 20.03$, $p= 0.002$ for females), there were no significant differences in performance between WT, HET and KO mice in either males or females (Figure S11).

***Nrxn1α* deletion was not associated with repetitive behaviours in mice**

Presence of repetitive behaviours was assessed in mice using the marble burying protocol. The numbers of marbles buried did not differ significantly between WT, HET and KO mice of either sex. The recording was checked, and marbles were counted at 10 minutes and 20 minutes to ensure that marbles were not buried and then unburied during the test (Table 4). Scores shown in Figure S12 were taken at the end of the task (at 30 minutes).

Table 4. Mean (± SEM) number of marbles buried by *Nrxn1α* mice at 10, 20 and 30 minutes.

	10 minutes	20 minutes	30 minutes
WT (M)	9 (1.0)	9 (0.4)	10 (0.5)
HET (M)	7 (0.7)	9 (0.6)	10 (0.3)
KO (M)	5 (1.1)	7 (1.3)	8 (1.5)
WT (F)	8 (0.7)	9 (0.4)	10 (0.5)
HET (F)	9 (0.7)	10 (0.4)	11 (0.3)
KO (F)	7 (1.8)	9 (1.8)	9 (1.6)

Discussion

Ultrasonic vocalisation testing revealed decreases in the number of pups' calls in *Nrxn1α* KO mice. The mean number of USVs was highest at PND 12 for each genotype and the greatest difference between the number of calls from KO mice compared to their WT and HET littermates was around PND 6. Moreover, the calls emitted by KO mice at PND 2, 4, 6 and 12 were of significantly shorter duration than the other genotypes. The sonographic profile of ultrasonic vocalisation also was altered in *Nrxn1α* mice. *Nrxn1α* KO mice emitted a restricted repertoire of calls when compared to WT and HET mice over the first two postnatal weeks, producing less *complex* and *two-components* calls and a higher number of *short* calls. These findings may be indicative of a loss in the complexity of USV production with *Nrxn1α* KO mice being less able to produce syllables with several internal frequency changes and preferring to emit calls with a short duration.

Many studies have looked into USV communication in mouse pups carrying deletions in genes or regions associated with ASD. *SHANK1* is a gene that encodes a synaptic scaffolding protein and has previously been associated with ASD (Leblond et al., 2014). Isolation-induced pup USVs were studied in *Shank1* (-/-) null mutant, *Shank1* (+/-) heterozygous, and *Shank1* (+/+) wildtype littermate controls between PND3 and 12. *Shank1* (-/-) pups were found to vocalise less than their littermates with a prominent genotype difference arising on PND 6, bearing similarity to the findings in the current study. The effects of social context also were tested in *Shank1* mutants using bedding from an unfamiliar adult conspecific, rather than clean bedding, in a second round of testing. The same genotype-dependent deficit was seen again (Sungur et al., 2016). Reductions in USV calls have also been seen in neuroligin mouse models including *Nlgn2* KO mouse pups (Wohr et al., 2013), mouse pups carrying the human R451C mutation in *Nlgn3* (Chadman et al., 2008) and a loss of function mutation in *Nlgn4* (Jamain et al., 2008). Conversely, pups on the BTBR genetic background called more loudly and frequently when separated from their litter in comparison to mice maintained on the C57BL/6J (B6) background (Scattoni et al., 2008). Mice on this background have previously shown autism-like behavioural deficits (McFarlane et al., 2008). Initially this difference was thought to be due to differences in body size, since the BTBR mice are larger and may therefore have larger thoracic sizes. However, when compared to other strains of mice with similar body weights, the abnormality persisted.

The early vocal repertoire has been also studied in many other ASD animal models from a qualitative point of view, detecting spectral and temporal properties of the calls and classifying them into different categories (Scattoni et al., 2008; Roy et al., 2012). Similar to *Nrxn1α* mice, Reeler mutant pups produced a narrowed repertoire of calls, specifically on PND 6 and 8, limiting their vocal repertoire to the *two-components*, *chevron*, and *complex calls*; while Dab1 mutant pups showed a

limited call pattern restricted to *short* and *downward calls* (Romano et al., 201; Fraley et al 2016). A poor USV repertoire also has been detected in *Cd157* KO pups from PND 3 until 10 compared to a rich repertoire in WT mice (Lopatina et al., 2017). These seemingly conflicting findings may simply indicate that the animal models reflect the diversity observed in patients across the different ASD, including the atypical vocalisations.

Concomitant with the assessment of vocal responsiveness, spontaneous motor behaviours have been assessed to evaluate motor coordination and balance skills acquired by pups during the first two postnatal weeks. In several ASD animal models, deficits in motor abilities have been detected in early development in addition to socio-communicative deficits (Michetti et al., 2017; Romano et al., 2013; Suetterlin et al., 2018, Whittaker et al., 2017). In the current study, *Nrxn1α* KO mice expressed an excessive pivoting behaviour at PND 2, 4 and 6 when they were partially able to move with the posterior forelimbs, as well as an increased ability to climb the wall of the glass container at PND 6, 8 and 12. Over the second postnatal week, pups start acquiring a better motor performance until they are able to fully move around the whole cage. At PND 12, *Nrxn1α* KO spent a lot of time walking and exploring the cage since they had increased locomotor activity and reduced immobility behaviour, suggesting they had a hyperactive motor profile compared to WT and HET pups. In line with these findings, the early behavioural characterization of *Chd8* HET mouse model described signs of abnormal motor development in the first two weeks after birth, including an increase in time spent moving compared to littermates, indicative of hyperactive spontaneous behaviour (Suetterlin P et al., 2018). However, when *Nrxn1α* KO were tested to detect subtle motor competences, they failed to show appropriate performances. Overall, the episodes of head shaking and head rising behaviours were reduced in *Nrxn1α* KO during PND 2 to 12. Face washing was reduced in *Nrxn1α* KO compared to WT and HET, particularly at PND 12, suggesting that *Nrxn1α* KO pups did not show a mature motor profile when they need to have an appropriate equilibrium. As *Nrxn1α* KO, Reeler pups showed a deficit in face washing and wall climbing at PND 12 compared to control mice. This and other studies carried out in ASD animal models, such as Reeler, Synapsin, CHD7 and CHD8 mice, showed a delayed or abnormal development of the motor system during the first 12 PNDs (Michetti et al., 2017; Romano et al., 2013; Suetterlin et al., 2018, Whittaker et al., 2017). Thus, it could be very useful to evaluate the neonatal motor profile in the context of behavioural phenotyping of ASD animal models, since early motor abnormalities also have been reported in infants and children diagnosed with ASD (Moseley and Pulvermüller, 2018). These motor alterations could have an effect on the acquisition of socio-communicative functioning and on other aspects of the development over postnatal life, resulting on deficits in the emission of ultrasonic vocalisations.

To further assess developmental effects in the *Nrxn1α* mutant, mice were tested through a battery of developmental milestones. In males, body weight, body and tail length were significantly lower in KO mice than WT and HET littermates from around PND 9 until the end of testing. These measures in females were unaffected by the *Nrxn1α* deletion. This could be associated with some of the other developmental delays seen in males in this part of the study. Both male and female ear opening was delayed in KO mice at PND 15; however, eye opening, fur appearance and incisor eruption were normal. Body weight and length are unlikely to be responsible since females did not show any delays in growth. Both male and female KO mice show significant delays grasping the vertical screen at PND 15 in which the pup must both grasp the screen and begin to climb. This may suggest reduced grasp reflexes; however, the horizontal screen and grasp reflexes measurements were unaffected. Body weight is also unlikely to have affected the finding since female body weight was not affected by *Nrxn1α* deletion making the cause of this delay unclear. Negative geotaxis was affected in male KO mice from PND 13 to PND 15, while KO females did not show any delays. In this case it is possible that the reduced male body weight may have been responsible. Male KO mice showed a severe deficit in cliff avoidance at PND 15. When placed on the apparatus, male KO mice appeared to charge off immediately, while HET and WT mice were able to reverse away from the edge, back to safety. Female KO mice also showed a trend towards this behaviour. Cliff avoidance is used as a measure of visual ability (Fox, 1965); however, given the odd behaviour of the KO mice in this task, it is unlikely that this task was providing an assessment of visual ability. Grayton et al. (2013) reported higher levels of anxiety in adult male mice. It is possible that a higher level of anxiety is also present in pups. If so, running off the apparatus may be an attempt to run away from the experimenter. Similar findings have been found in other studies of developmental milestones. NLGN2 is a member of the neuroligin family of proteins that form trans-synaptic complexes with neurexins and, like *Nrxn1α*, is associated with both ASD and schizophrenia (Sun et al., 2011; Reissner et al., 2013; Parente et al., 2017). Homozygous *Nlgn2* KO mice were tested between PND 2 and PND14 and showed delays in certain developmental milestones including shorter body length, later eye opening, later incisor eruption and reduced grasp reflex. As was seen in our study, these mice showed no delay in righting reflex or acoustic startle. By adulthood, these measures were normal (Wohr et al., 2013). A study of mouse pups with the human R451C mutation in Nlgn3 revealed minor developmental differences in comparison to the WT, including slightly different growth rates and slower righting reflexes at postnatal days 2-6 (Chadman et al., 2008). Unlike the findings in our study, developmental milestones and growth rates were accelerated in a study of the BTBR mouse (Scattoni et al., 2008). The mice were tested between PND2 and PND14 and had significantly higher body weight and tail length and earlier eye opening and incisor eruption than B6 mice. They also showed earlier completion of forelimb grasp

reflex, vertical screen and cliff aversion tests. However, they showed delays in negative geotaxis and righting reflex (the former of which was also seen in the *Nrxn1α* KO mice). It is possible that their larger size could have accounted for some of these differences and the earlier eye opening may account for the earlier cliff aversion.

This study characterised the behaviour of juvenile and adult *Nrxn1α* KO mice in detail, using mice at earlier developmental stages than previously described and on mice backcrossed onto a single genetic background (C57BL/6J). Adult male KO mice show significantly increased levels of aggression and abnormalities in social behaviour. These findings replicate those in Grayton et al., 2013. Social behaviours could be analysed and interpreted as they were presented, since olfactory ability in mice was not a confounding factor. Previously, a buried cookie test has shown that olfactory deficits are not present in *Nrxn1α* KO mice (Grayton *et al*., 2013), but findings from this test may differ from those in the olfactory habituation protocol given that the buried cookie test can be influenced by the mouse's appetitive behaviour (Gusmão et al., 2012). The olfactory habituation test performed during this study confirmed that there are no olfactory deficits in HET and KO *Nrxn1α* mice. During the juvenile play protocol, male KO and HET mice showed a significant reduction in investigative behaviours only, which persisted into adulthood. Analysis of the individual investigative behaviours revealed that the grouped behaviours were significant mainly due to sniffing duration times. This is particularly compelling since sniffing behaviour is an active behaviour in which mouse contact occurs, while the following and mutual circle behaviours do not necessarily involve contact. Therefore, a clear decrease in active contact is observed in KO mice. In adulthood, KO mice also showed decreased investigative and affiliative behaviours and high levels of aggression in the social interaction test. Increases in aggression were present in the HET mice as well as the KO mice, suggesting a strong association with *Nrxn1α* and aggressive behaviours. The presence of increased aggression and decreased investigative behaviours in HET mice is interesting since it shows evidence of a heterozygote effect, which is particularly important when considering that the majority of *NRXN1* deletions in human patients are found in the heterozygous condition. However, the decreased investigative behaviours that are present in juvenile HET mice were no longer present in adulthood. It is possible that having one functioning *Nrxn1α* gene could compensate for the loss of the other copy as mice enter adulthood, meaning that changes to affiliative and investigative behaviours are not seen (although aggression does appear). Overall these findings suggest that in males the deleterious effects of *Nrxn1α* on behaviour are present in juveniles and become more severe in adulthood and in the homozygous condition, providing evidence for the role of *Nrxn1α* in aggressive behaviours in both mice and humans.

One possibility for the adult behavioural abnormalities such as aggression seen in male mice could be that they are a consequence of early social dysfunction. Similarly, human literature discusses how early frustration in children with ASD who cannot interact with other children can result in certain behaviours such as avoidance or venting and tension releasing behaviours (both verbal and non-verbal) (Diener and Mangelsdorf, 1999; Jahromi et al., 2012). Female behaviour appeared to be unaffected by *Nrxn1α* deletion during these two tests. Although no significance or trends were reached, the graph profiles appear to show a slight decrease in investigative and affiliative behaviours in juvenile, but not in adult female mice. As in the previous study by Grayton et al. (2013), there were no differences in the level of aggression seen in adult female KO mice. Differences in aggression exist between male and female mice (St John and Corning, 1973; vom Saal et al., 1976; Parmigiani et al., 1998), with males tending to show higher levels of aggression towards intruder mice than females. Female mice typically only show aggression to intruders during lactation and rearing pups (Parmigiani et al., 1998). It would be interesting to study the social behaviour of female HET and KO mice with offspring in a future study. Social abnormalities were also observed during the three-chamber social approach testing, however some of the findings indicated that the protocols had not worked successfully and cannot be fully interpreted. Two protocols were run, a juvenile three chamber social approach and an adult version that was modified so that the test mice were under the wire cup as a "novel mouse" and the arena was explored by conspecifics. No previous publications have used the three-chamber social approach test in juveniles or with these modified positions, so it is possible these tests are not robust in mice and validation of the use in juveniles and modified 3-chamber task are warranted.

In addition to social behaviours, repetitive behaviours and motor learning behaviours were assessed. Adult body weight was found to be lower in male and female *Nrxn1α* KO mice. This could potentially confound locomotor activity and should be considered when interpreting results of these tests. The rotarod is a method used to assess motor learning and co-ordination in mice (Wohr et al., 2013). There were no differences in rotarod performance between genotypes in either sex. Therefore, on the pure genetic background, the *Nrxn1α* deletion was not associated with deficits in motor learning. However, it has previously been reported that on the mixed genetic background *Nrxn1α* KO mice exhibit enhanced motor learning on the rotarod (Etherton et al., 2009). Etherton et al. (2009) also observed increases in repetitive behaviour measured by observing the number of self-grooming bouts and time spent self-grooming in *Nrxn1α* KO mice. In both the marble burying protocol in this study and the self-grooming assessment by Grayton et al. (2013) using a pure genetic background, genotype effects on repetitive behaviour was not seen. Further to this, when looking at the data profile, the male KO mice appear to bury less marbles than their WT and HET littermates. Following

this finding, the video recording was checked at 10 and 20 minutes to ensure that mice were not burying and then uncovering marbles but still no significant differences were seen. We can conclude from this study and that by Grayton et al. (2013) that *Nrxn1α* deletion is not associated with repetitive behaviour. Although repetitive behaviour is seen in many patients with ASD (DSM-V, American Psychiatric Association, 2013) the fact it is not seen in this study does not mean the *Nrxn1α* mouse cannot be used as a model of ASD. Mouse models are unlikely to reproduce every possible feature of a disorder given ASD are complex disorders and not due to variation in one gene.

As this study presents evidence of a developmental trajectory for mouse behaviour, future research using this mouse model should aim to characterise animal behaviour at younger juvenile ages than described here. The first step that should be taken into account is to perform the juvenile play task at P21 rather than P30. In this study P30 was chosen so that tests could be run at the same time points as those in Grayton et al. (2013), since our study aimed to replicate a number of the findings from Grayton's paper. Ideally, we would like to run the task at even younger ages, however, running social tests below P21 becomes difficult since rodents younger than P21 do not tend to exhibit fully developed exploratory behaviours (Barnett, 1958). In addition, if performing testing before weaning it would be difficult to establish whether observed behavioural abnormalities were related to the mother or to pup-pup interactions.

In conclusion, we present evidence for a role of *Nrxn1α* deletion in social behaviours, one of the core symptom domains affected in ASD. This suggests that deletions within the NRXN1 gene in patients could be responsible for the social impairments. The *Nrxn1α* deletion also causes developmental delays in young mice aged from approximately PND 9 to PND 15, reduced and restricted vocal repertoire, as well as early motor abnormalities. Though there were no repetitive behaviours present, the purpose of mouse models is not to directly mimic every symptom associated with a disorder. Furthermore, this is the first study to describe a developmental trajectory in *Nrxn1α* KO mice, since many earlier studies have focused on adult mice. The deleterious effects of *Nrxn1α* deletion have been shown to be present in juvenile mice but become more severe in adulthood, while in females this is not the case. It is well known that more males than are affected by ASD in the human population (Werling and Geschwind, 2013). The reasons for this apparent "protective effect" in females are unknown and could be explored in this mouse model since it appears to reflect the sex differences seen in humans.

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Assessing the developmental trajectory of mouse models of neurodevelopmental disorders: social and communication deficits in the Neurexin 1α Knockout Mouse.

SUPPLEMENTAL MATERIAL

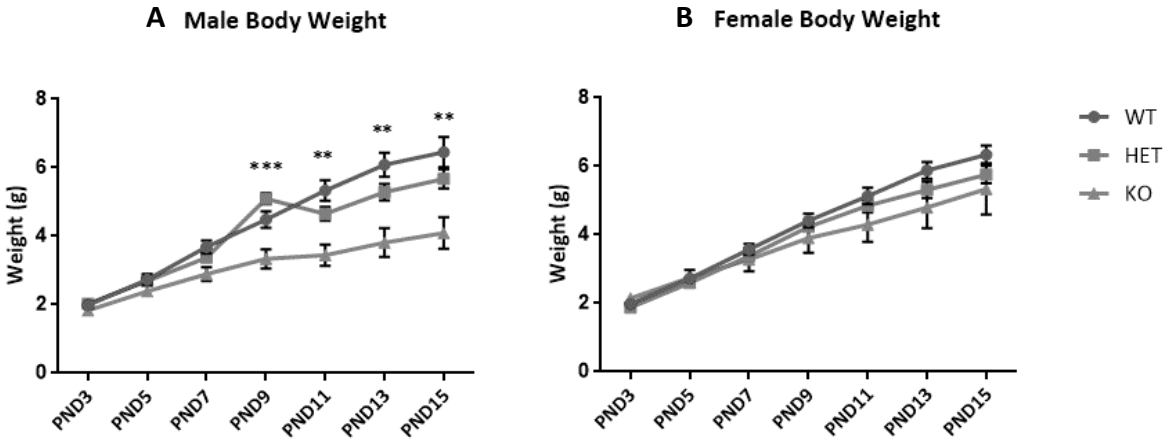

Figure S1. Mean (± SEM) body weight (g) measurements from PND3 to PND15 in male (A) and female (B) *Nrxn1α* mice. Data derived from 8 WT, 24 HET, 7 KO males and 16 WT, 21 HET, 6 KO females**.** Levels of significance indicated by *** $p < 0.001$ and ** $p < 0.01$, compared to WT mice.

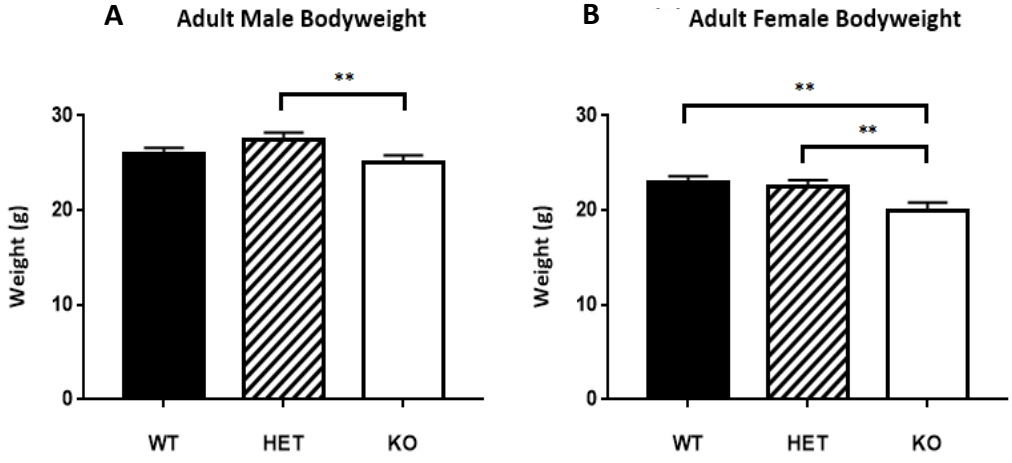

Figure S2. Mean (± SEM) body weight (g) of the male (A) and female (B) adult *Nrxn1α* mice. Derived from 12 WT, 15 HET, 9 KO males and 11 WT, 14 HET, 9 KO females. Levels of significance indicated by ** $p < 0.01$, compared to WT mice.

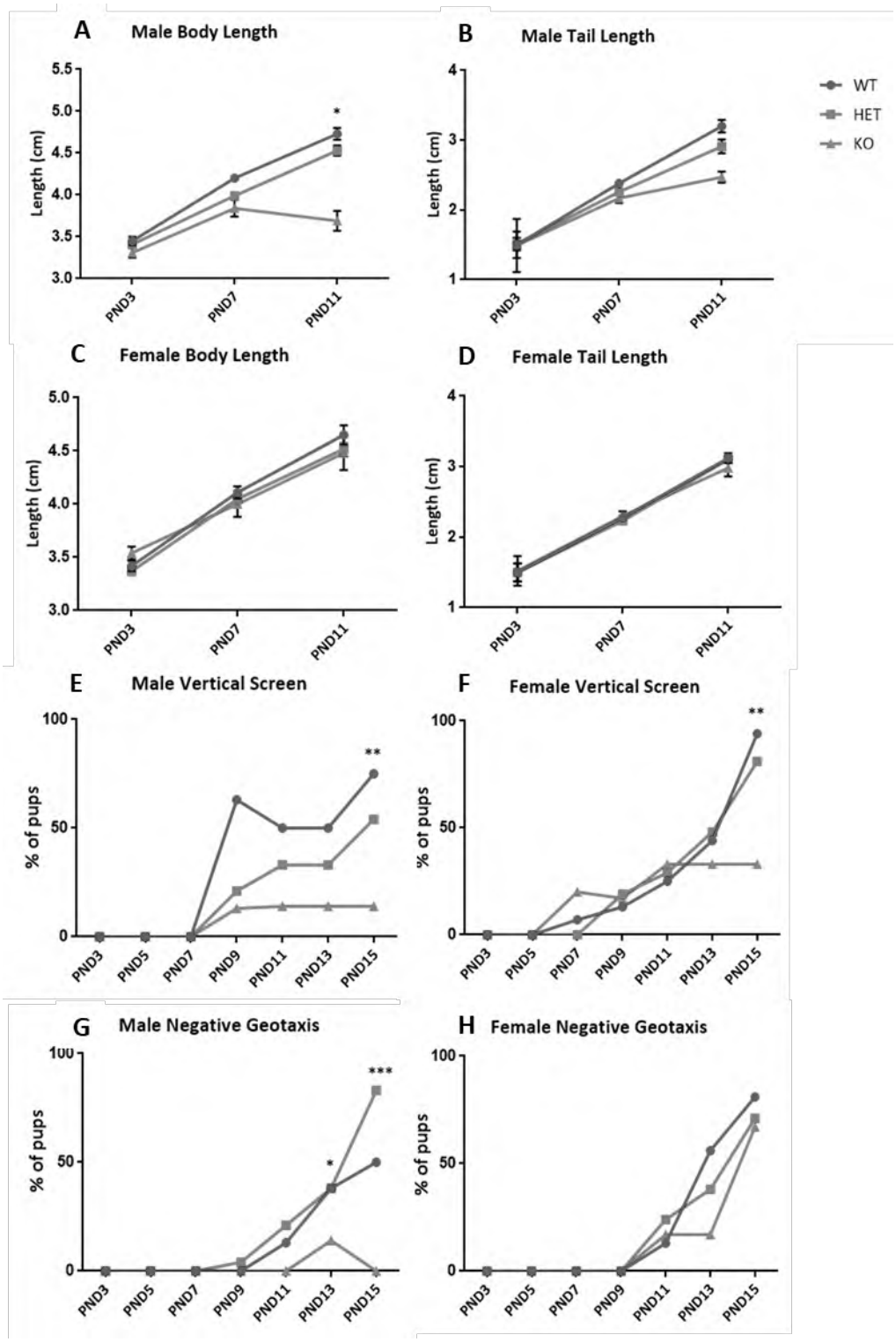

Figure S3. Mean (± SEM) body length (A, C), tail length (B, D), grasping (% of pups to attain the maximum score at each post-natal day) during the vertical screen test at PND 15 (E, F), mean (± SEM) negative geotaxis (G, H) in male and female *Nrxn1α* mice. Data derived from 8 WT, 24 HET, 7 KO males; 16 WT, 21 HET, 6 KO females. Levels of significance indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT mice.

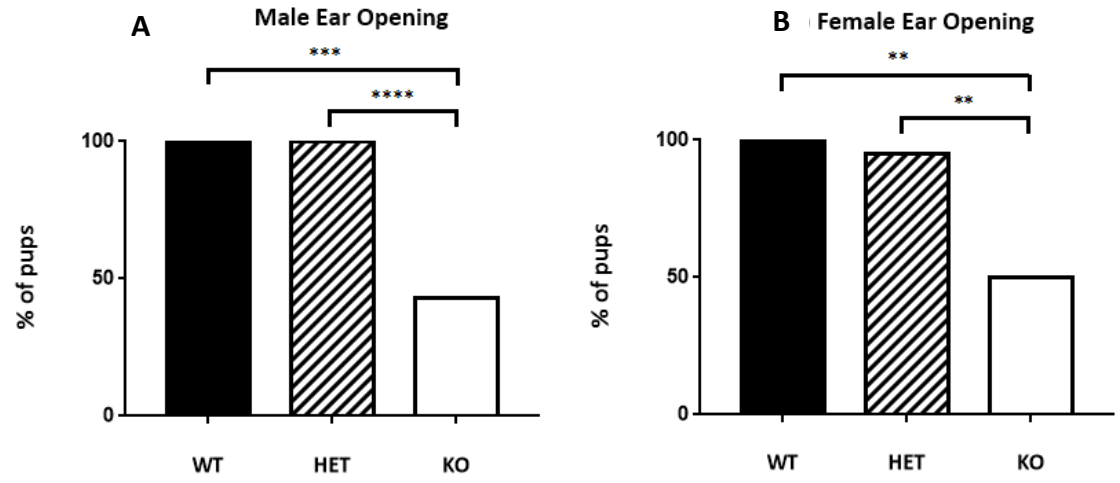

Figure S4. Male (A) and female (B) ear canal opening (% of pups to attain the maximum score) in *Nrxn1α* mice at PND 15. Data derived from 8 WT, 24 HET, 7 KO males and 16 WT, 21 HET, 6 KO females. Levels of significance indicated by ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, compared to WT mice.

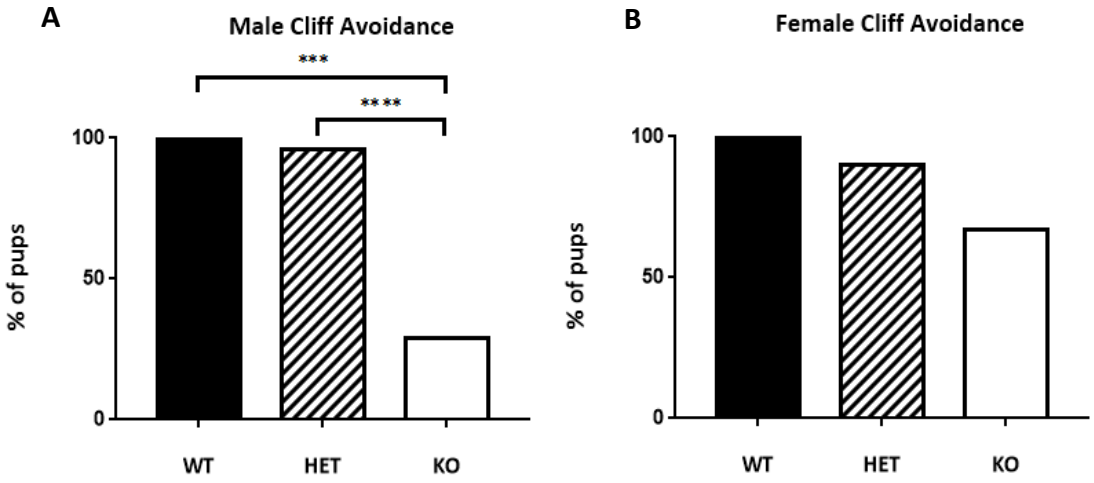

Figure S5. Cliff avoidance (% of pups to attain the maximum score) in male (A) and female (B) *Nrxn1α* mice at PND 15. Data derived from 8 WT, 24 HET, 7 KO males and 16 WT, 21 HET, 6 KO females. Levels of significance indicated by *** $p < 0.001$ and **** $p < 0.0001$, compared to WT mice. Females show a trend only (0.06).

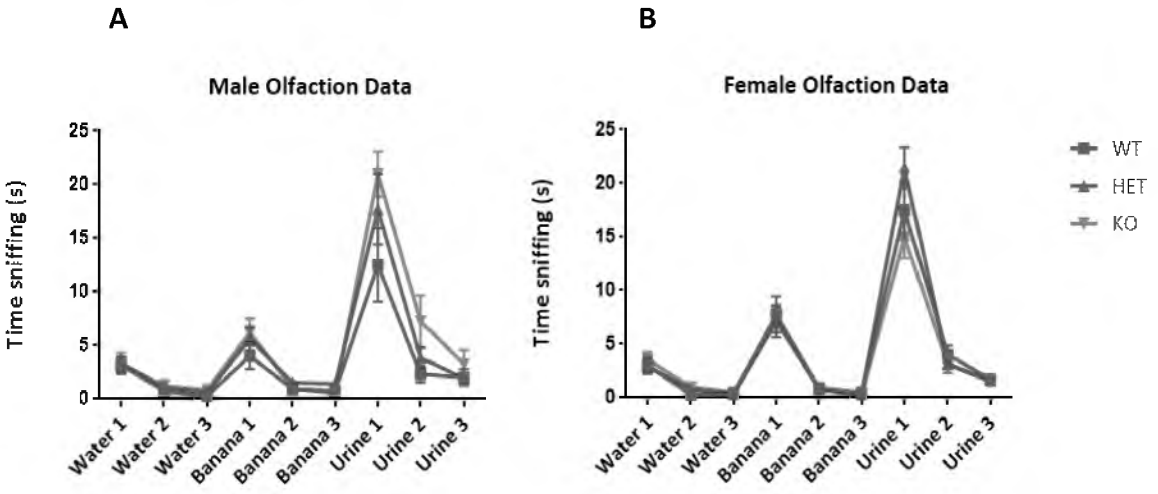
A
Male Olfaction Data
Time sniffing (s)
25
20
15
10
5
0
Water 1
Water 2
Water 3
Banana 1
Banana 2
Banana 3
Urine 1
Urine 2
Urine 3
B
Female Olfaction Data
Time sniffing (s)
WT
HET
KO

Figure S6. Olfactory habituation/ dishabituation in adult male (A) and female (B) *Nrxn1α* mice. Data derived from 6 WT, 8 HET and 8 KO males and 8 WT, 7 HET and 8 KO females. Each point represents mean (± SEM) time sniffing (s) on exposure to each odour.

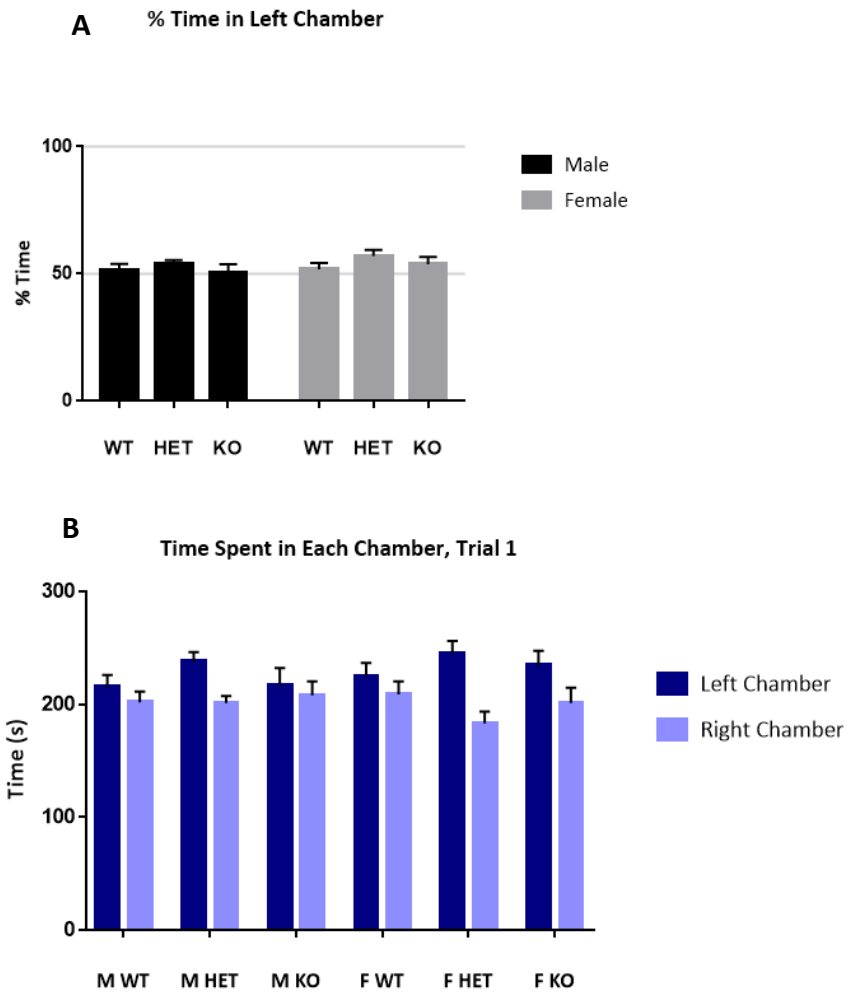

Figure S7. Three chamber social approach task in juvenile *Nrxn1α* mice on Trial 1. Mean (± SEM) % time spent in the left chamber (A), where 50% represents the chance level. Profile of time spent in left, centre and right chamber (B). Data derived from 12 WT, 19 HET and 19 KO males and 16 WT, 18 HET and 15 KO females.

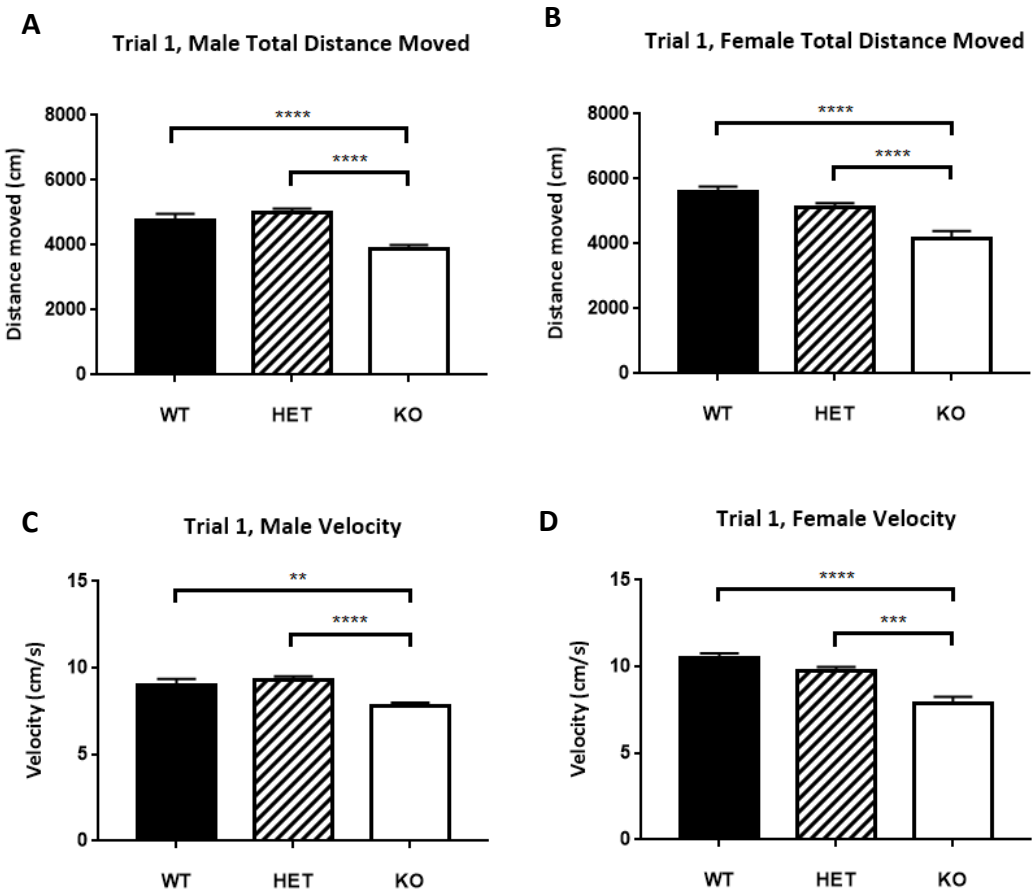

Figure S8. Mean (± SEM) total distance moved by (A, B), and total velocity (C, D) of, male and female juvenile *Nrxn1α* mice during Trial 1 of the 3-chamber social approach task. Data derived from 12 WT, 19 HET and 19 KO males and 16 WT, 18 HET and 15 KO females. Levels of significance indicated by ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

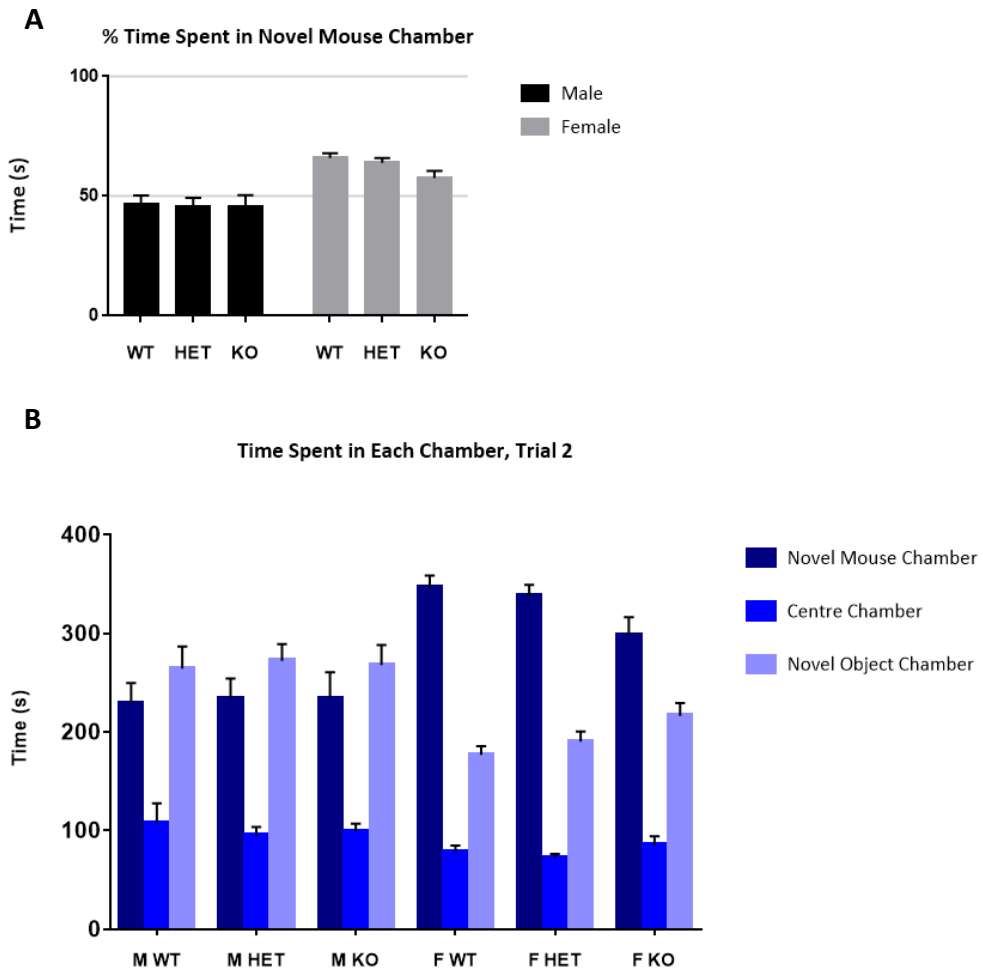

Figure S9. Three chamber social approach task in juvenile *Nrxn1α* mice, trial 2. Mean (± SEM) % time spent in the novel mouse chamber (A), where 50% represents the chance level. Profile of time spent in left, centre and right chamber (B). Data derived from 12 WT, 19 HET and 19 KO males and 16 WT, 18 HET and 15 KO females.

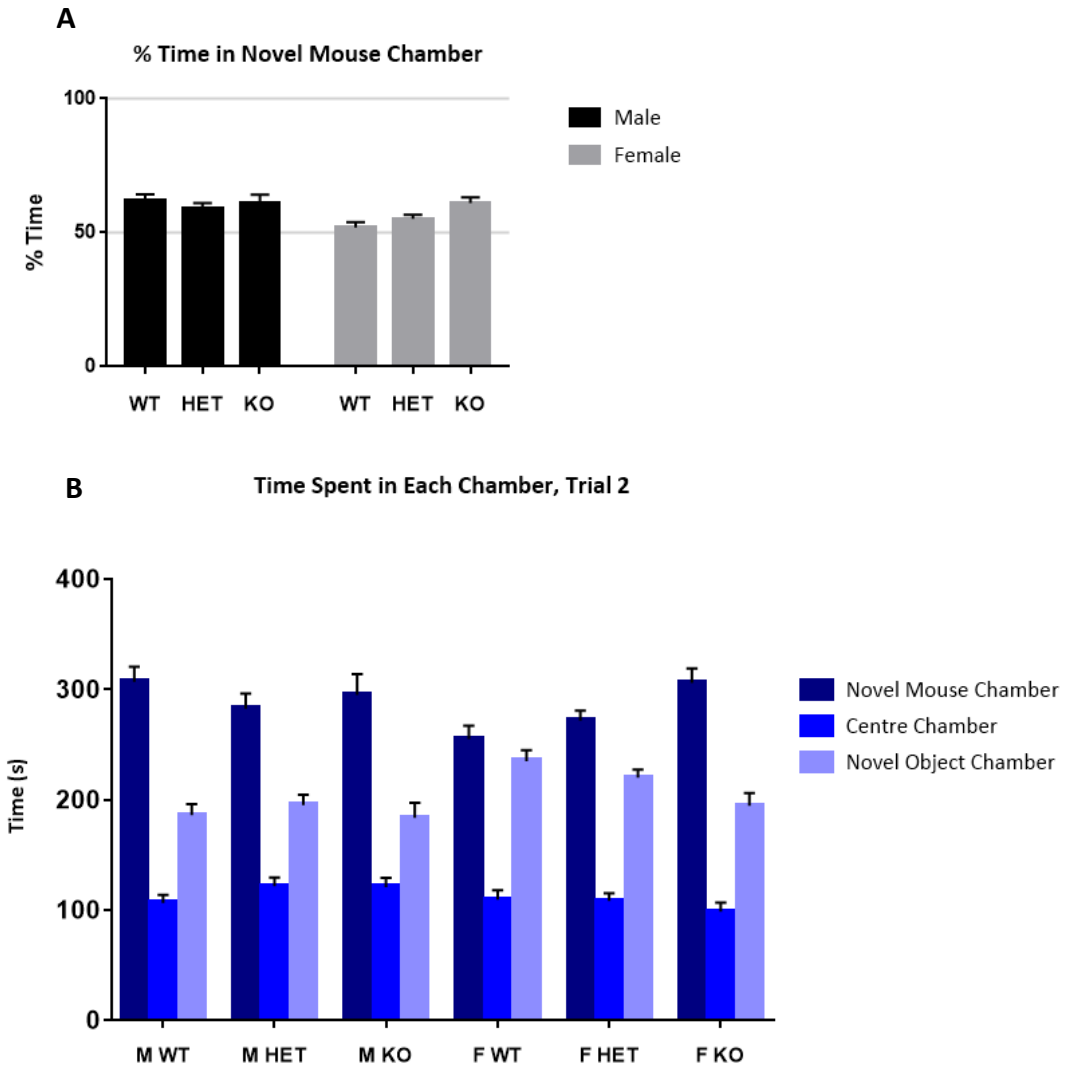

Figure S10. Modified three chamber social approach task for adult *Nrxn1α* mice, with reversed positions, in Trial 2. Mean (± SEM) % time spent in the novel mouse (WT/HET or KO) chamber (A), where 50% represents the chance level. Profile of time spent in novel mouse, centre and novel object chamber (B). Data derived from 16 WT, 18 HET and 15 KO females and 12 WT, 19 HET and 19 KO males.

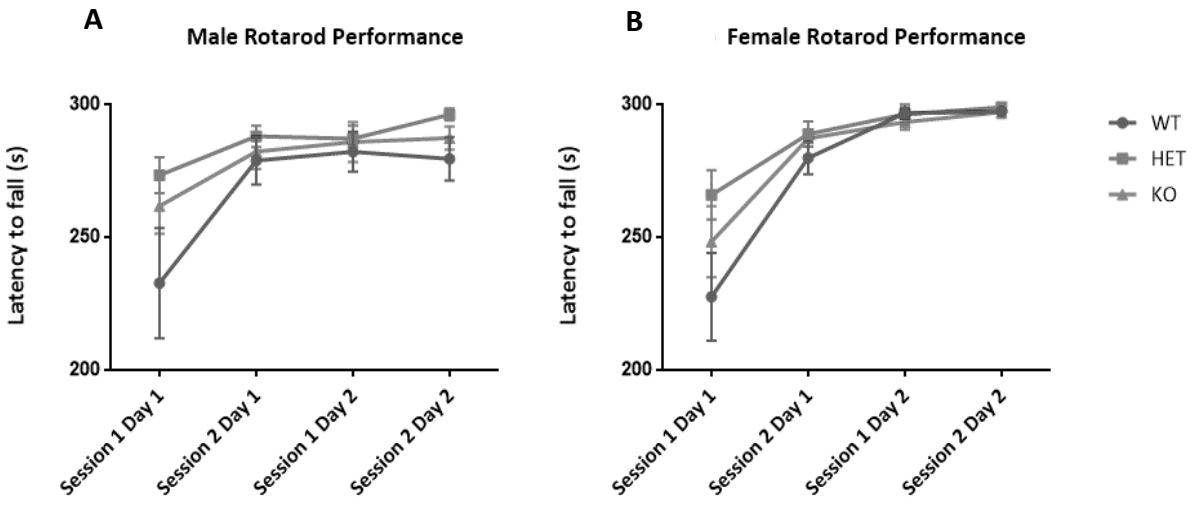

Figure S11. Mean (± SEM) rotarod performance in male (A) and female (B) juvenile *Nrxn1α* mice. Data derived from 13 WT, 20 HET and 18 KO males and 16 WT, 19 HET and 14 KO females.

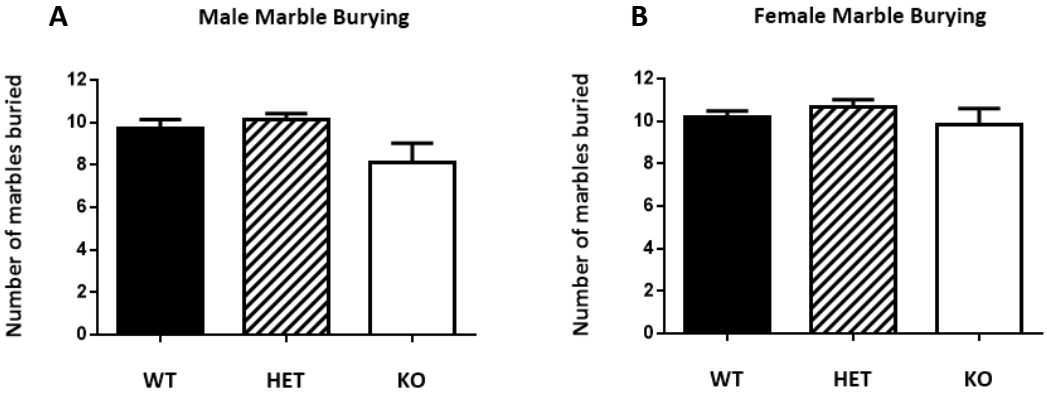

Figure S12. Mean (± SEM) marble burying scores after 30 minutes in male (A) and female (B) adult *Nrxn1α* mice show no differences between genotypes. Data derived from 13 WT, 20 HET and 18 KO males and 16 WT, 19 HET and 14 KO females.